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The Uronic Acids in a Hydrolyzate of
Sapote Gum

Roger D. Lambert

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THE URONIC ACIDS IN A HYDROLYZATE OF SAPOTE GUM

A thesis submitted by

Roger D. Lambert

B.S. in Pulp and Paper Technology 1961,
North Carolina State College
M.S. 1963, Lawrence College

in partial fulfillment of the requirements
of The Institute of Paper Chemistry
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Appleton, Wisconsin

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SUMMARY

The compositions of five nodules of each of two samples of sapote gum were determined. The ten nodules contained units of D-xylose, L-arabinose, uronic acid, and methoxyl in an average molar ratio of 2.2:1.0:1.0:0.58. Only minor quantitative inter- and intrasample variations were found. Chromatographic evidence indicated that the uronic acid units were D-glucuronic acid and 4-O-methyl-D-glucuronic acid. Associated with the sapote gum was approximately one percent of a galactan-rich neutral polysaccharide.

Two aldotriouronic acids, 4-O-methyl-D-glucopyranosyluronic acid(1 $\xrightarrow{\alpha}$ 2)D-xylopyranose(1 $\xrightarrow{\beta}$ 4)D-xylopyranose (A) and D-glucopyranosyluronic acid(1 $\xrightarrow{\alpha}$ 2)D-xylopyranose(1 $\xrightarrow{\beta}$ 4)D-xylopyranose (B), were isolated, by preparative paper chromatography, from a partial acid hydrolyzate of sapote gum. (A) has been isolated from several sources, but apparently (B) has not been reported previously. Paper chromatographic evidence indicated that partial acid hydrolysis probably also produced aldotetraouronic acids.

(A) was identified by comparison with an authentic specimen (K). (A) and (K) had identical x-ray diffraction patterns and, within experimental accuracy, identical equivalent weights, specific rotations, chromatographic mobilities, and melting points. (B) was identified by: (1) the identification of D-glucopyranosyluronic acid(1 $\xrightarrow{\alpha}$ 2)D-xylopyranose (as the crystalline acetylated methyl ester, methyl glycoside) as a hydrolysis product of (B), (2) the identification of 1,2,3,5-tetra-O-methyl-D-xylitol (as the crystalline p-nitrobenzoate) as a hydrolysis product of the neutral, methylated, nonreducing derivative of (B), and (3) the similarity of the specific rotations of (B) and (A), which, according to the rules of isorotation, indicated that the configurations of the two glycosyl bonds in (B) were dissimilar.

The methylated, nonreducing derivatives of (A), (K), and (B) were found to have, within experimental accuracy, identical infrared absorption spectra and specific rotations. The acid hydrolysis products of the neutral, methylated, non-reducing derivatives of (A), (K), and (B) corresponded, by paper chromatography (relative mobility), to 2,3,4-tri-O-methyl-D-glucose and 3,4-di-O-methyl-D-xylose and, by gas chromatography (retention time), to 1,2,3,5-tetra-O-methyl-D-xylitol. The methanolizate of the neutral, methylated, nonreducing derivatives of (A), (K), and (B) contained (as was indicated by gas chromatography) five products. There were no intersample variations in the relative retention times of these products. Presumably, there were 1,2,3,5-tetra-O-methyl-D-xylitol, the α and β anomers of methyl 3,4-di-O-methyl-D-xylopyranoside, and the α and β anomers of methyl 2,3,4-tri-O-methyl-D-glucopyranoside.

INTRODUCTION

Sapote gum is available commercially from Peru where it is collected from trees of the genus Sapota. The trees, which grow at approximately 3000 feet elevation in the semitropical climate near the equator, are tapped for latex from which chicle is prepared. The gum slowly covers the wounds after the flow of latex ceases (1). The composition and structure of this gum have been studied by Anderson and Ledbetter (2) and by White (3).

Anderson and Ledbetter (2) found crude sapote gum exudate to consist of 90 to 94% water-soluble acidic polysaccharide and 6 to 10% water-insoluble resin. The resin was presumed to be latex which became mixed with the polysaccharide during gum formation. In the remainder of the present writing the term "sapote gum" will refer only to the polysaccharide portion of the exudate.

Anderson and Ledbetter (2) found sapote gum to consist of units of uronic acid, D-xylose, and L-arabinose, and of methoxyl groups. They found approximately 1 methoxyl group to 2 uronic acid units and 7 pentose units. The pentose was found to consist of approximately 1 L-arabinose unit to 8.5 D-xylose units. The composition of the gum acid is given in Table I.

TABLE I
COMPOSITION OF SAPOTE GUM ACID^a (2)

Uronic acid, %	27.51
Pentosan, %	67.9
CH ₂ , from methoxyl, %	1.30
Total, %	96.7
Pentose units per uronic acid	3.61
Methoxyl unit per uronic acid	0.65

^a

Results corrected for moisture and ash.

The presence of a uronic acid unit was established by a naphthoresorcinol test and by the evolution of carbon dioxide when the gum was heated in a 12% solution of hydrochloric acid. Although the uronic acid was not fully identified, these workers stated that they found indications of D-glucuronic acid but none of D-galacturonic acid. The presence of a methoxyl group ether-linked to some of the uronic acid residues was established by the methods of Deniges (4) and von Fellenberg (5). D-xylose and L-arabinose were isolated from the acid hydrolyzate of the gum and identified. Tests for hexoses and methylpentoses were negative.

Anderson and Ledbetter (2) also studied the structure of sapote gum by acid hydrolysis techniques. The composition of the barium salts from each hydrolyzate which they produced is given in Table II.

As is shown in Column 1 of Table II, after mild hydrolysis the barium salts contained 2.31 pentose units per uronic acid unit. Evidence was obtained which indicated that all of the L-arabinose had been liberated and that only D-xylose units remained attached to the uronic acid units.

The composition of salts from two samples hydrolyzed under somewhat more severe conditions are given in Columns 2 and 3 of Table II. The two samples differed only in that the sample in Column 3 was fractionated more than the sample in Column 2 when the barium salts were separated from the neutral sugars by precipitation with ethanol. These data show that approximately two D-xylose units per uronic acid unit were left after hydrolysis. This ratio of 2:1 is, of course, the ratio for neutral sugar to uronic acid in an aldotriouronic acid. The ratio of 0.61 methoxyl units per uronic acid unit was very similar to the ratio of 0.65 for the free sapote gum acid (Table I).

TABLE II

BARIUM SALTS FROM HYDROLYSIS OF SAPOTE GUM^a (2)

	Col. 1 Salts from Gum Heated for 2 Hr. at 80°	Col. 2 and 3 Salts from Gum Heated for 4 Hr. at 80°	Col. 4 Salts from Barium Salts 1, 2, and 3 Heated in Autoclave for 4 Hr. at 120°	Col. 5 Salts from Gum Heated for 28 Hr. in Boiling Water Bath
Barium, %	13.14	14.43	14.20	23.43
Pentosan, %	55.5	50.5	52.6	26.1
Uronic acid, %	35.02	37.08	36.42	51.54
CH ₂ from methoxyl, %	--	--	1.60	2.11
Total, %	--	--	104.82	103.18
Pentose units per uronic acid	2.31	1.98	2.1	0.74
Methoxyl unit per uronic acid	--	--	0.61	0.564

^a In all cases, a 4% solution of sulfuric acid was used for hydrolysis.

The results of severe acid hydrolysis conditions are given in Columns 4 and 5 of Table II. Even under these conditions some of the D-xylose units remained attached to the uronic acid units, suggesting the formation of a resistant aldobiouronic acid. From the decreased values of the ratio of methoxyl to uronic acid, it can probably be assumed that methoxyl was cleaved from some of the uronic acid groups.

White (3) studied the composition and structure of the methyl ether derivative of sapote gum. Extraction of a saponified methanolizate of this derivative with anhydrous ether separated the soluble neutral glycosidic components from the insoluble uronosidic components. Fractional distillation of the neutral glycosides yielded methyl glycosides of 3-O-methyl-D-xylose; 2,3,4-tri-O-methyl-D-xylose; and 2,3,4-tri-O-methyl-L-arabinose.

A second uronosidic fraction was prepared from saponified methanolizate by absorption of the uronosides upon ion-exchange resin, elution, and subsequent re-esterification. From this fraction was obtained, by fractional distillation and hydrolysis, 3,4-di-O-methyl-D-glucopyranosyluronic acid and what appeared to be a mixture of two aldobiouronates. Repeated methanolysis of this mixture and subsequent hydrolysis produced some 3-O-methyl-D-xylose. Apparently, this was the only neutral sugar unit present in the aldobiouronic acids, and hydrolysis severe enough to release it probably destroyed any monomeric uronic acids which were released.

A third uronosidic fraction was prepared in the same manner as described for the first fraction, with the ether-insoluble acidic components being retained. A portion of this fraction which boiled over the boiling range of the methyl ester methyl glycoside of 3,4-di-O-methyl-D-glucuronic acid (which had been identified previously) was discarded, as was a still residue. The portion remaining was reduced to give nonacidic materials. Some of this reduced product was hydrolyzed and yielded 3-O-methyl-D-xylose; 3,4-di-O-methyl-D-glucose; and 2,3,4-tri-O-methyl-D-glucose. Presumably, these sugars arose from two partially methylated aldobiouronic

acids, one containing 3,4-di-O-methyl-D-glucuronic acid and 3-O-methyl-D-xylose and one containing 2,3,4-tri-O-methyl-D-glucuronic acid and 3-O-methyl-D-xylose. Another portion of the reduced product was methylated and hydrolyzed to yield 3,4-di-O-methyl-D-xylose and 2,3,4,6-tetra-O-methyl-D-glucose. Evidently, the aldobiouronic acid (or acids) present before reduction had eventually been converted by reduction and methylation to a single product, 2,3,4,6-tetra-O-methyl-D-glucose(1 \rightarrow 2)3,4-di-O-methyl-D-xylose.

Quantitative paper chromatography of a hydrolyzed portion of methanolized sapote gum methyl ether, using the previously identified partially methylated sugars as standards, gave the following molar ratio of products: 3-O-methyl-D-xylose, 2.80 parts; 3,4-di-O-methyl-D-glucuronic acid determined as 3,4-di-O-methyl-D-glucose, 1.11 parts; 2,3,4-tri-O-methyl-D-glucuronic acid determined as 2,3,4-tri-O-methyl-D-glucose, 1.07 parts; 2,3,4-tri-O-methyl-L-arabinose, 1.05 parts; and 2,3,4-tri-O-methyl-D-xylose, 0.97 part. White stated that these figures represented, within experimental accuracy, a molar ratio of 3:1:1:1, although he emphasized the complexity of the evaluation and the possibility for error.

White summarized the above results concerning the structure of the sapote gum molecule with the aid of a repeating unit concept. He emphasized, however, that there is, as yet, insufficient evidence to support the hypothesis of a definite, uniformly reproduced, repeating system as constituting the structure of any heteroglycan. He concluded that the main chain structure must consist largely, if not entirely, of D-xylose units. Two-thirds of these D-xylose units must be linked in the main chain with glycosidic 1 \rightarrow 4 bonds since D-glucuronic acid is attached to them at C₂ and a free hydroxyl is located on C₃. He assumed that the other one-third of the D-xylose units in the main chain are also joined with glycosidic 1 \rightarrow 4 bonds to the rest of the chain. Each of the D-xylose units in the main chain must have a side chain at C₂; one-third of these side chains must consist of a

unit of either D-xylose or L-arabinose, while the other two-thirds must contain D-glucuronic acid units. Some of the D-glucuronic acid units must have D-xylose or L-arabinose units attached at C_2 . From the evidence of Anderson and Ledbetter (2) White concluded that approximately half of the D-glucuronic acid units contain a methoxyl group, and, by analogy with other gums, he assumed that the methoxyl is probably attached at C_4 .

Smith and Montgomery (1) have interpreted White's conclusions in the form of a structural formula for a repeating unit of sapote gum (Fig. 1). This structure adheres to White's conclusions except that certain restrictions which cannot be substantiated by the data are introduced. The configuration of none of the glycosyl bonds is known, and there is no valid reason for placing a methoxyl group on the uronic acid unit which is a complete side chain and none on the other uronic acid unit. Without evidence to the contrary, a random distribution of methoxyls must be assumed. A different arrangement of the uronic acid units could have been suggested in which these units were not on adjacent xylose units.

It must be emphasized that this repeating unit is only a working concept, and that other structures could be suggested which would explain the data equally well. For instance, side chains could be longer, and the different side chains could be randomly distributed.

In addition to the studies described above, three other instances are known to the author in which the compositions of substances thought to be sapote gum have been investigated. Dutton, in a personal communication to Smith and Montgomery (1), reported a composition of D-glucuronic acid (17%), L-arabinose (25.1%), D-galactose (45.5%), L-rhamnose (6.8%), D-xylose (5.2%), and methoxyl (2%) for a sample of gum which was "obtained through commercial channels from Peru and sold as sapote gum." A white powder which the author obtained commercially in the

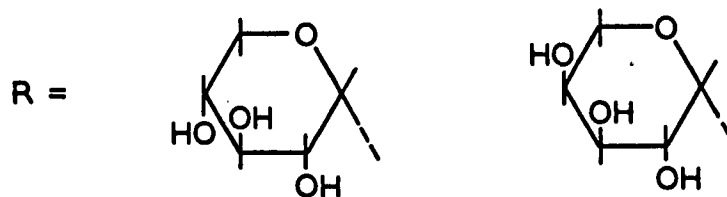
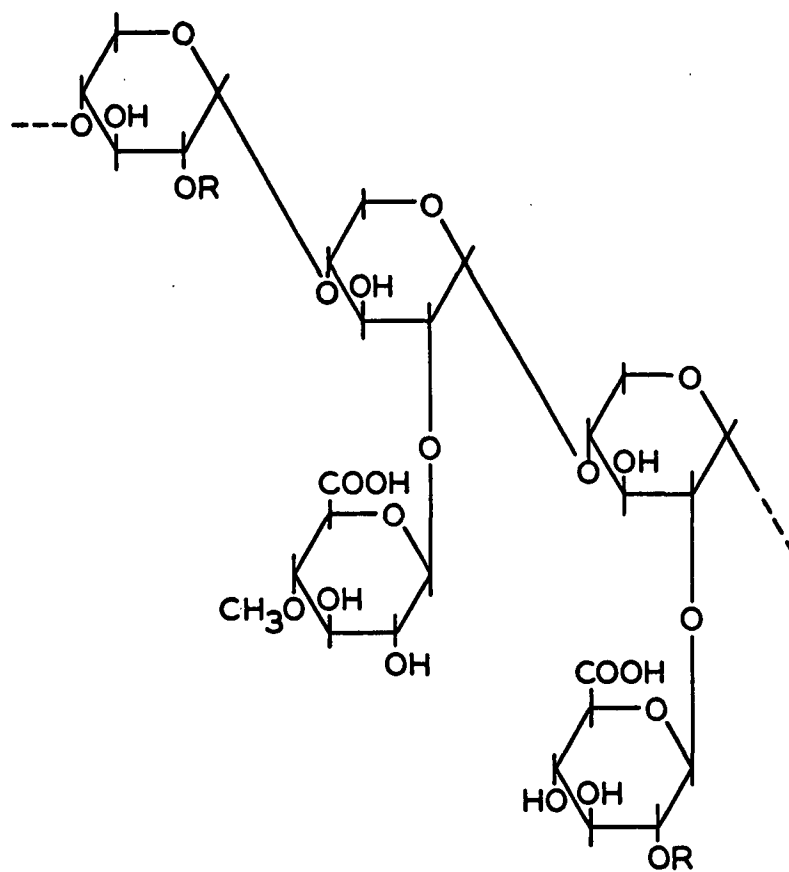


Figure 1. A Hypothetical Repeating Unit of Sapote Gum (1)

United States was called sapote gum and contained these approximate percentages of residues: uronic anhydride (25%), galactan (37%), araban (21%), xylan (7%), mannan (5%), and methoxyl (4%). Another sample which was obtained by The Institute of Paper Chemistry contained the following approximate percentages of residues: uronic anhydride (27%), galactan (20%), araban (21%), xylan (25%), mannan (2%), and methoxyl (2.6%). From the compositions of these samples it is apparent that either the compositions of various samples of sapote gum vary considerably or materials which have been obtained commercially have been incorrectly identified.

ANALYSIS OF THE PROBLEM

The first objective in the present study was the obtaining of evidence which might indicate whether different samples of sapote gum differ greatly in composition. Considerable doubt existed concerning the composition of sapote gum because several investigators, each working with a different sample of material thought to be sapote gum, had obtained compositions which disagreed both quantitatively and qualitatively with respect to one another. Thus, it was necessary to obtain information regarding the probable reasons for these disagreements before any very meaningful conclusions could be drawn from information concerning the structure of the sapote gum molecule.

The primary objective in the present study was the identification of some of the oligouronide fragments produced by partial acid hydrolysis of sapote gum and the relation of these fragments to the structure of the sapote gum molecule.

Evidence obtained by Anderson and Ledbetter (2) indicated that under rather severe acid hydrolysis conditions two aldobiouronic acids are produced, one consisting of units of a uronic acid (probably D-glucuronic acid) and D-xylose and the other consisting of units of a monomethyl uronic acid and D-xylose. By analogy with other gums they assumed that the monomethyl uronic acid is probably 4-O-methyl-D-glucuronic acid.

White (3) found that the uronic acid units in sapote gum are indeed units of D-glucuronic acid and that they are joined by glycosyl bonds to C₂ of D-xylose units. The configuration of this bond was not determined, nor did White's study provide additional information concerning the location of the methoxyl group which Anderson and Ledbetter had found to be associated with some of the uronic acid units. White obtained evidence (but not proof) that the D-xylose units to which uronic acid units are attached are part of a main chain of D-xylose units joined by glycosyl 1→4 bonds.

By relatively mild acid hydrolysis of sapote gum, Anderson and Ledbetter (2) obtained a mixture of acidic materials, an analysis of which indicated that oligouronic acids of higher degree of polymerization than aldobiouronic acids were probably present. The presence of 0.6 mole of methoxyl per mole of carboxyl indicated that some of the oligouronic acids probably contained a methoxyl substituent and that others probably did not.

In unpublished studies, Thompson (6) and Dickey (7) subjected partial acid hydrolyzates of sapote gum to qualitative paper chromatographic analysis. They obtained several chromatographic spots of acidic materials, which indicated that aldouronic acids of higher degree of polymerization than aldobiouronic acids probably had been produced. One of these acids corresponded to the well-known aldotriouronic acid 4-O-methyl-D-glucopyranosyluronic acid(1^α→2)D-xylopyranose-(1^β→4)D-xylopyranose.

The results of these studies suggested an interesting hypothesis that two series of aldouronic acids might occur in the partial acid hydrolyzate of sapote gum, the acids of one series consisting of units of D-glucuronic acid and D-xylose and the acids of the other series consisting of units of a monomethyl (probably 4-O-methyl-) D-glucuronic acid and D-xylose.

The specific objectives of the present study were:

1. The obtaining of information concerning possible differences between the compositions of various samples of sapote gum.
2. The determination of whether two series of oligouronic acids are produced by partial acid hydrolysis of sapote gum.
3. The isolation of one member of each series of oligouronic acids found.
4. The determination of the composition and structure of each of the aldouronic acids isolated.

5. The elucidation of the probable relationships between the aldouronic acids characterized and the structure of the sapote gum molecule.

EXPERIMENTAL RESULTS

Two samples of sapote gum were available. One sample, the "old" gum had been at The Institute of Paper Chemistry for several years and was believed to be part of the sample which had been studied previously by White (3). The other sample, the "new" gum, was obtained in 1964 from Exportadora El Sol S.A., Apartado 2404, Lima, Peru. This gum was believed to have come from trees of the species Sapota achras, but the possibility that other species of the genus Sapota were involved cannot be eliminated completely. Each sample consisted of a large number of nodules which had not been processed chemically, mechanically, or in any other manner since their collection. The nodules of the "old" sample were uniformly dark brown. Many of the nodules of the "new" sample were also dark brown, but others were various lighter shades of brown. Pieces of twigs and bark were imbedded in many of the nodules of both samples.

COMPOSITION OF INDIVIDUAL NODULES

The compositions of five nodules of each of the two samples of gum were determined. Three objectives were intended: (1) the determination of whether the two samples differed significantly in composition, (2) the determination of the amount of variation in composition of nodules in each sample, and (3) a comparison of the compositions of these two samples with the compositions of samples of sapote gum described in the literature.

PURIFICATION OF GUM

Five nodules from the "old" sample were selected at random. Five nodules from the "new" sample were selected to represent the range of color (light to dark brown) of this sample. Each nodule was dissolved in 500 ml. of distilled water to give a brown solution from which pieces of bark and wood were removed

by filtration. To each solution were added 5 ml. of 10% sodium chlorite and 0.5 ml. of glacial acetic acid. When the brown color had disappeared, each solution was poured into 1500 ml. of 95% ethanol, whereupon the gum precipitated as white, gelatinous flocks. The liquid phase was decanted, and the precipitates were solvent-exchanged five times with absolute ethanol, three times with anhydrous ethyl ether, and twice with petroleum ether. The precipitates were allowed to air dry from the petroleum ether.

COMPLETE ACID HYDROLYSIS

Fifty mg. of each of the ten samples of gum were dissolved in 17 ml. of 8.5% sulfuric acid and were heated at approximately 115°C. for 60 min. The hydrolyzates were cooled, neutralized with barium carbonate, and filtered through a cellulose mat on a vacuum filter. The filtrates were evaporated to dryness at approximately 30°C. with a rotary vacuum evaporator.

PAPER CHROMATOGRAPHY

Two drops of water were added to each of the ten dried hydrolyzates. Portions of the ten solutions were spotted on several sheets of Whatman No. 1 chromatographic paper, along with authentic samples of L-rhamnose, D-xylose, L-arabinose, D-ribose, D-glucose, D-mannose, D-galactose, D-glucuronic acid, and 4-O-methyl-D-glucuronic acid. All of the chromatograms were developed by the descending solvent technique. One group of the chromatograms was developed with Solvent [A]¹ (ethyl acetate:pyridine:water; 8:2:1), which caused only neutral sugars to migrate; one group was developed with Solvent [B] (ethyl acetate:acetic acid:water; 9:2:2), which caused both neutral and acidic sugars to migrate; another group was developed first with

¹
All solvents and detecting reagents are described in Appendix I.

Solvent [B] and then with Solvent [A], which caused neutral sugars to drip from the chromatograms and left only acidic sugars. (Throughout the present study the effects of these two solvents upon various substances were used as criteria for determining whether such substances were neutral or acidic.) Some of the chromatograms from each group were sprayed with a p-anisidine hydrochloride spray which gives different colors for pentoses, hexoses, and uronic acids (8). The other chromatograms from each group were dipped in a silver nitrate solution which gives black spots for pentoses, hexoses, and uronic acids, but which is more sensitive than the p-anisidine hydrochloride spray (8). The silver nitrate dip gave spots corresponding in migration rate to D-xylose, L-arabinose, D-galactose, D-glucuronic acid, and 4-O-methyl-D-glucuronic acid. The p-anisidine hydrochloride spray revealed the same spots, and, in addition, imparted to each spot a color which was the same as the color of the authentic substance of equal migration rate. The spot which corresponded to D-galactose was faint on all chromatograms which were not spotted very heavily, indicating that the substance which it represented was present in each hydrolyzate in a much smaller quantity than the substances represented by the other spots.

SUGAR, URONIC ANHYDRIDE, AND METHOXYL ANALYSES

Portions of each of the ten purified samples of gum were analyzed for sugar anhydrides (9), uronic anhydride (Institute Procedure 25), and methoxyl (TAPPI Method T 209 m-45). As shown in Table III, the compositions of the ten samples were very similar. No significant differences between the "old" and the "new" samples were apparent from these analyses. The average molar ratio of xylan, araban, uronic anhydride, and methoxyl was 2.2:1.0:1.0:0.58.

Approximately 1.0% of galactan was found, but evidence obtained by Dr. N. S. Thompson (10) indicated that galactan is not part of the sapote gum molecule. One gram of the "new" gum was dissolved in water, and three grams of diethylaminoethyl

cellulose were stirred into the solution. Subsequent washing of the diethylamino-ethyl cellulose and evaporation of the filtrate gave 30 mg. of residue. After it was hydrolyzed, this material was inspected by paper chromatography. The most abundant substance present corresponded to galactose. Smaller spots corresponded to glucose and mannose. No spots corresponding to xylose or arabinose were present. Apparently, this material was a small amount of hemicellulose or other polysaccharide which was associated with the sapote gum.

TABLE III
COMPOSITION OF GUM NODULES^a

Nodule ^b	Galactan, %	Araban, %	Xylan, %	Methoxyl (as CH ₂), %	Uronic Anhydride, % ^c	Total, %
N1	1.00	22.0	45.6	1.30	27.6	97.5
N2	0.93	25.4	44.4	1.06	26.1	97.9
N3	0.82	20.2	47.8	1.23	28.1	98.1
N4	1.03	22.2	43.5	1.46	28.6	96.8
N5	1.05	24.6	45.1	1.27	26.5	98.6
Average	0.97	22.9	45.3	1.26	27.4	97.8
O1	1.07	18.1	46.0	1.56	28.0	94.8
O2	0.55	24.6	45.8	1.19	26.8	99.0
O3	0.74	21.8	44.2	1.38	27.4	95.5
O4	1.04	21.7	45.9	1.25	26.7	96.6
O5	1.68	20.0	46.8	0.98	28.1	97.6
Average	1.02	21.2	45.7	1.27	27.4	96.7
Grand average	1.00	22.1	45.5	1.27	27.4	97.3

^a
All values are corrected for moisture and ash.

^b
N refers to "new" gum, O to "old" gum.

^c
By evolution of CO₂.

LARGE-SCALE PURIFICATION OF "NEW" GUM

A portion of the "new" gum was purified in the manner described previously for the purification of the ten nodules. Nodules (150 g. total) were selected at random and were placed in a glass funnel which was suspended just beneath the surface of ten liters of distilled water in a battery jar (7). As the gum dissolved, concentrated solution sank to the bottom of the jar and less concentrated solution flowed into the top of the funnel. All of the gum eventually dissolved in this manner.

The solution was filtered to remove pieces of bark and wood. Half of a 125-ml. aqueous solution containing ten grams of sodium chlorite, 5 ml. of glacial acetic acid, and 1 ml. of formic acid was added to the brown solution of gum. About five hours later the rest of the bleaching solution was added (at approximately 25°C., the temperature during the entire chloriting procedure). Two days later the gum solution was colorless except for a yellowish cast caused by chlorine dioxide. One hundred ml. of 37% hydrochloric acid (diluted to approximately 250 ml.) were added to the solution. One-half hour later the solution was poured into 30 liters of 95% ethanol, whereupon the gum precipitated as a white gelatinous flock. The liquid phase was removed by decantation and filtration, the precipitate was washed with 95% ethanol, and dissolved in three liters of distilled water. Thirty ml. of hydrochloric acid (diluted to approximately 100 ml.) were added to the solution, and after 45 min. the solution was poured into nine liters of 95% ethanol, which caused the gum to precipitate. The liquid phase was removed by decantation and filtration. The precipitate was washed thoroughly with absolute ethanol, and finally with anhydrous ethyl ether. The gum was spread on a watch glass and was allowed to air dry overnight. The yield was approximately 96 g.; the percentage of ash was negligible; moisture was 9.85%.

QUALITATIVE ANALYSIS OF "NEW" GUM

Complete and partial acid hydrolyzates of the purified "new" gum were investigated by paper chromatography. The objective was the tentative determination of the nature of the oligouronic acids produced by partial acid hydrolysis.

ACID HYDROLYSIS

Two 50-mg. samples of the purified "new" gum were hydrolyzed. Sample I was dissolved in 17 ml. of 8.5% sulfuric acid and was heated at approximately 115°C. for 60 min. Sample II was dissolved in 20 ml. of 0.5N sulfuric acid and was heated at approximately 115°C. for 50 min. The hydrolyzates were allowed to cool to room temperature. Sulfate ion was removed by precipitation with barium carbonate and vacuum-filtration through a cellulose mat. The filtrates were evaporated to dryness at approximately 30°C. with a rotary vacuum evaporator and were stored over phosphorous pentoxide in a vacuum desiccator.

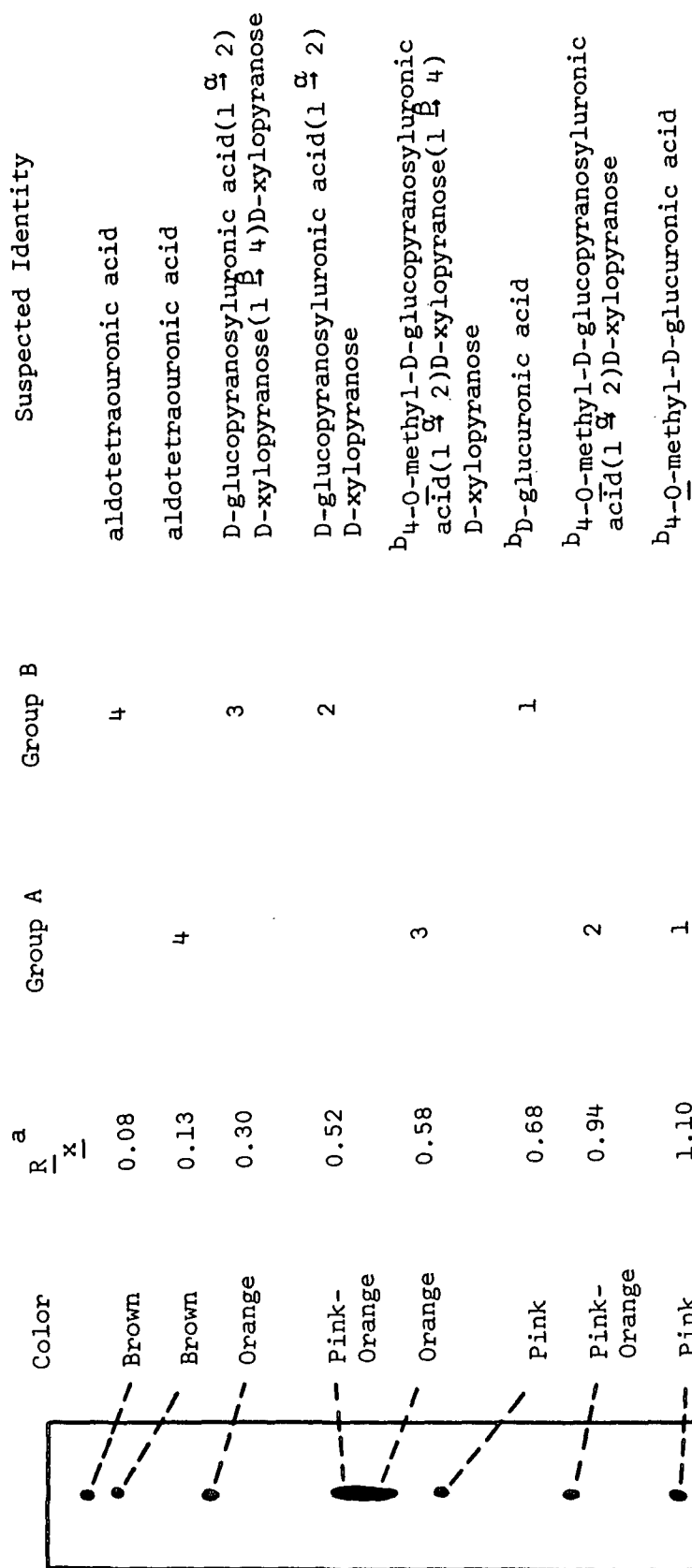
PAPER CHROMATOGRAPHY

The hydrolyzates of Samples I and II were chromatographed by the descending solvent technique on Whatman No. 1 chromatography paper with Solvents [A], [B], and [B] followed by [A]. The substances which migrated were detected with silver nitrate dip and with p-anisidine hydrochloride spray.

Apparently, Sample I had been hydrolyzed completely to monomers. The products from this sample seemed to be the same as the products from the ten nodules described previously; three neutral materials corresponded in migration rate and color (with p-anisidine hydrochloride spray) to authentic samples of D-xylose, L-arabinose, and D-galactose; two acidic substances corresponded in migration rate and color (bright pink) to authentic samples of D-glucuronic acid and 4-O-methyl-D-glucuronic acid. The identity of the D-glucuronic acid was very questionable on

the basis of these chromatograms because D-galacturonic acid migrates at almost the same rate as D-glucuronic acid in Solvent [B]. However, further evidence that one of the acids was D-glucuronic acid was obtained when the hydrolyzate was rechromatographed after it had been allowed to evaporate slowly to dryness; a spot of a neutral substance corresponding to D-glucurone was then observed. D-glucuronic acid is known to form D-glucurone readily, but D-galacturonic acid does not readily form a lactone.

Five spots from Hydrolyzate II corresponded to the five spots from Hydrolyzate I, but, in addition, six other spots of acidic materials were observed on chromatograms of Hydrolyzate II. The identities of these six spots were not known immediately, but it could be assumed that they almost certainly were oligouronic acids. As was discussed previously (p. 12), there was reason to expect two series of oligouronic acids in the partial acid hydrolyzate of sapote gum, one series consisting of acids containing residues of D-glucuronic acid and D-xylose, and the other series consisting of acids containing residues of 4-O-methyl-D-glucuronic acid and D-xylose. An examination of Fig. 2 shows that the eight acidic spots from Hydrolyzate II could be grouped into four pairs on the basis of color; that is, there were two spots each of pink, pink-orange, orange, and brown. On this basis the spots were divided into two groups, the faster spot of each color in Group A and the slower spot in Group B. Thus, the first spot in Group A corresponded to 4-O-methyl-D-glucuronic acid and the first spot in Group B corresponded to D-glucuronic acid. It was then tentatively assumed that Group A contained aldouronic acids which contained residues of 4-O-methyl-D-glucuronic acid and that Group B contained aldouronic acids which contained residues of D-glucuronic acid. Support for this assumption was provided by the fact that the second and third spots, respectively, of Group A corresponded in migration rate and color to spots of authentic 4-O-methyl-D-glucopyranosyluronic acid (1 $\xrightarrow{\alpha}$ 2)D-xylopyranose and 4-O-methyl-D-glucopyranosyluronic acid (1 $\xrightarrow{\alpha}$ 2)D-xylopyranose (1 $\xrightarrow{\beta}$ 4)D-xylopyranose.



^a Mobility relative to D-xylose, with Solvent [B].

^b Probable identity indicated by chromatographic comparison with authentic material.

Figure 2. Aldouronic Acids from Partial Acid Hydrolyzate of Sapote Gum

Further evidence that the spots in Groups A and B represented two series of aldouronic acids is presented in Fig. 3. $\underline{R_m}$ is plotted against \underline{n} , the number of monomeric units (suspected) in each acid to give similar curves for Groups A and B. French and Wild (11) have shown that for a homologous series such as the xylo-dextrins or cellodextrins a plot of this type is linear when

$$R_m = \log (1 - R_f)/R_f$$

and $\underline{R_f}$ is the distance migrated by a particular substance divided by the distance moved by the solvent front. For Fig. 3, $\underline{R_m}$ was calculated from the equivalent equation

$$R_m = \log (1 - cR_x)/cR_x$$

in which \underline{c} is the distance migrated by xylose divided by the distance moved by the solvent front and $\underline{R_x}$ is the distance migrated by a particular substance divided by the distance migrated by xylose.

For Groups A and B the curves approach linearity as \underline{n} increases, as would be expected since, as \underline{n} increases, the migration rate of the aldouronic acids should be affected less by the presence of the single carboxyl group. It probably is also significant that as \underline{n} increases the two curves seem to be converging. Since the two series of acids are thought to differ only in the presence of a 4-O-methoxyl group on the acids of one series, the two curves would be expected to converge as \underline{n} increases and the effect of the methoxyl group upon chromatographic mobility decreases.

On the basis of the evidence cited it was tentatively assumed that two monomeric uronic acids and two series (each containing three members) of oligouronic acids were present in the partial acid hydrolyzate of sapote gum. A correlative assumption was that the second member of each series of oligouronic acids was an

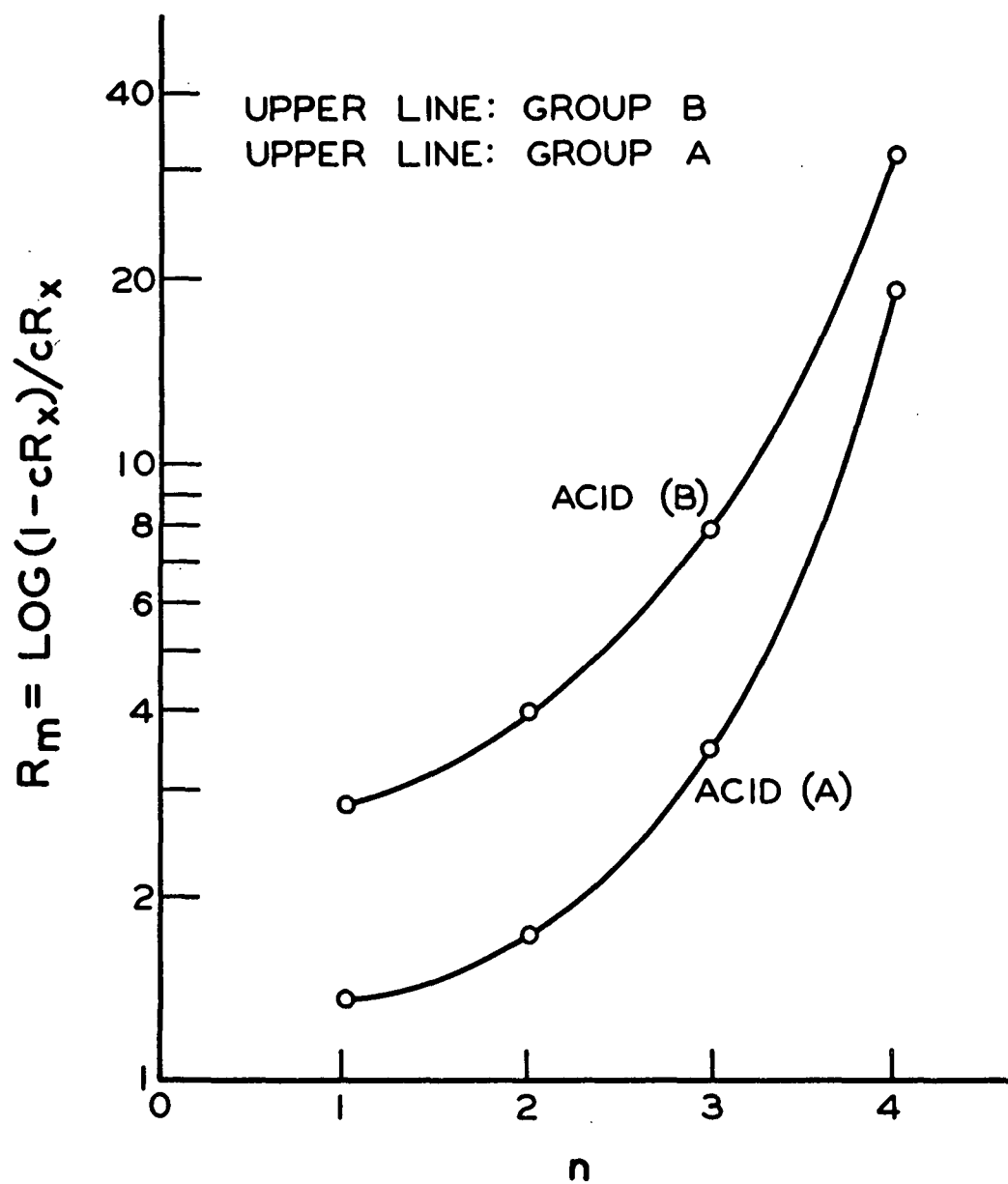


Figure 3. Chromatographic Mobility of Acids from Partial Acid Hydrolyzate of Sapote Gum

aldotriouronic acid [Acids (A) and (B) in Fig. 3]. One of these acids, Acid (A), corresponded in migration rate and color to 4-O-methyl-D-glucopyranosyluronic acid (1 α 2)D-xylopyranose(1 β 4)D-xylopyranose. It was reasonable to assume that, if Acid (A) really was this well-known aldotriouronic acid, then Acid (B) was probably the related aldotriouronic acid D-glucopyranosyluronic acid(1 α 2)D-xylopyranose(1 β 4)D-xylopyranose since White (3) had discovered previously that the uronic acid residues in sapote gum are probably joined by glycosyl bonds to D-xylose residues in a main chain of 1-4 linked D-xylose residues.

ISOLATION AND IDENTIFICATION OF D-XYLOSE AND L-ARABINOSE

Four 2.0-g. samples of the purified "new" gum were each dissolved in 300 ml. of 8% sulfuric acid and were hydrolyzed completely by being heated at 115°C. for 120 min. The hydrolyzates were neutralized with barium carbonate, filtered, combined, and evaporated to a thin sirup. Approximately one half of the sirup was placed on five 11-1/4-inch-wide sheets of Whatman No. 17 chromatography paper. The sheets were irrigated for 30 hours with Solvent [A], dried, and inspected by the peel-strip method. Strips containing suspected D-xylose and L-arabinose were cut from the chromatograms. The sugars were eluted from these strips with distilled water. The two solutions were passed through columns of Amberlite IR-120(H⁺) and IR-45(OH⁻) ion-exchange resins and then were evaporated to thick sirups which were placed over phosphorus pentoxide in a vacuum desiccator. The L-arabinose crystallized overnight, and the next day a few seed crystals were added to the D-xylose, which was then placed on a rotary vacuum evaporator (at room temperature) until, after a short while, the D-xylose had crystallized.

The D-xylose was identified as the dibenzylidene dimethyl acetal derivative (12,13). From a 0.425-g. sample of the suspected D-xylose, 0.540 g. of the derivative (78% of theoretical yield) was obtained. From a 0.452-g. sample of authentic

D-xylose, processed simultaneously, a yield of 0.559 g. (75% of theoretical yield) was obtained. The two products were found to melt at 208°C., alone or in admixture with an authentic sample.

The L-arabinose was identified by a comparison of its infrared absorption spectrum with that of an authentic sample. This comparison was considered sufficient for identification since both White (3) and Anderson and Ledbetter (2) had previously identified L-arabinose from sapote gum.

ISOLATION AND CHARACTERIZATION OF TWO ALDOTRIOURONIC ACIDS

The two suspected aldotriouronic acids were isolated, by preparative paper chromatography, from a partial acid hydrolyzate. Several chemical and physical properties of each acid were determined.

PARTIAL ACID HYDROLYSIS

Twelve grams of the purified new gum were placed in each of eight beakers. To each sample were added 250 ml. of 0.25N sulfuric acid, and the samples were heated at approximately 115°C. for 50 min. The hydrolyzates were neutralized, while hot, with barium carbonate, vacuum-filtered through cellulose mats, combined, and concentrated to 200 ml.

PARTIAL SEPARATION OF NEUTRAL SUGARS FROM OLIGOURONIC ACIDS

The barium salts of the aldouronic acids were precipitated by the addition of 300 ml. of absolute ethanol. After three hours, during which the solution was shaken occasionally, the alcohol was decanted and saved (Solution A). The precipitate was dissolved in 300 ml. of distilled water, and the solution was acidified with dilute sulfuric acid. A small amount of barium sulfate formed and was removed

by vacuum-filtration through Celite¹ filter aid. An excess of lead acetate was added to remove sulfate ion, the lead sulfate being removed by filtration through Celite. Hydrogen sulfide was bubbled into the solution to precipitate excess lead ion as lead sulfide which was removed by filtration through Celite. The total volume of solution was now 1300 ml.

Ten percent (130 ml.) of the solution was added to 300 ml. of distilled water. To this solution were added 100 g. of Darco G-60 activated charcoal, and the slurry was stirred for two hours. The charcoal was removed by filtration on Celite, and the filtrate was saved (Solution B). The filter cake was washed with 1000 ml. of distilled water (Solution C), and 1500 ml. of 50% aqueous ethanol (Solution D), the two washings being saved separately. Solutions A, B, C, and D were evaporated to thick sirups which were investigated by paper chromatography. Sirup A contained only neutral sugars; Sirups B and C were almost identical to each other and contained neutral sugars and monomeric acids; Sirup D contained neutral sugars, monomeric acids, and oligouronic acids.

The other ninety percent of the hydrolyzate was processed in the manner described above. The aqueous ethanol washing yielded 10.1 g. of sirup rich in oligouronic acids. This sirup was placed over phosphorus pentoxide in a vacuum desiccator where it crystallized, after several days, as a light brown substance.

PREPARATIVE PAPER CHROMATOGRAPHY²

The crystalline material was dissolved in a few milliliters of distilled water and was placed on 24 sheets (22-1/2 inches wide) of Whatman No. 17 chromatography

¹
A description of all trade-marked items used is given in Appendix II.

²
The techniques mentioned here are described in Appendix I.

paper which had been washed with Solvent [B]. The sheets were irrigated for five days with Solvent [B] and then were air dried. Inspection of the chromatograms by the peel-strip technique (14) showed that the two suspected aldotriouronic acids had separated. Strips were cut from the chromatograms and the two acids were eluted with distilled water. The two solutions were passed through columns of Amberlite IR-120(H⁺) ion-exchange resin and were evaporated to thick, slightly yellowish sirups which were placed over phosphorus pentoxide in a vacuum desiccator. Approximately three days later the acids had crystallized. Acid (A), suspected 4-O-methyl-D-glucopyranosyluronic acid(1 $\xrightarrow{\alpha}$ 2)D-xylopyranose(1 $\xrightarrow{\beta}$ 4)D-xylopyranose, weighed 2.81 grams. Acid (B), suspected D-glucopyranosyluronic acid(1 $\xrightarrow{\alpha}$ 2)D-xylopyranose-(1 $\xrightarrow{\beta}$ 4)D-xylopyranose, weighed 1.20 grams. Qualitative paper chromatography with Solvents [A] and [B] indicated that the two acids were chromatographically pure.

CHARACTERIZATION OF ALDOTRIOURONIC ACIDS

Several chemical and physical properties of the two suspected aldotriouronic acids were determined. An authentic sample, Acid (K), of 4-O-methyl-D-glucopyranosyluronic acid(1 $\xrightarrow{\alpha}$ 2)D-xylopyranose(1 $\xrightarrow{\beta}$ 4)D-xylopyranose was used as a standard for comparison during some of the determinations.

Hydrolysis Products

A few milligrams of each of the two suspected aldotriouronic acids were completely hydrolyzed. Paper chromatography revealed one neutral and one acidic product in each of the hydrolyzates. The products from Acid (A) corresponded to 4-O-methyl-D-glucuronic acid and D-xylose. Those from Acid (B) corresponded to D-glucuronic acid and D-xylose.

Small portions of the two suspected aldotriouronic acids were partially hydrolyzed. A few milligrams of each acid were dissolved in 10 ml. of 0.5N sulfuric acid and were heated at approximately 115°C. for 50 min. Paper chromatography

revealed one neutral and three acidic materials from each hydrolyzate. In both cases the neutral substance corresponded to D-xylose. The acidic substances from Acid (A) corresponded, in decreasing order of migration rate, to 4-O-methyl-D-glucuronic acid, 4-O-methyl-D-glucopyranosyluronic acid(1 α 2)D-xylopyranose, and 4-O-methyl-D-glucopyranosyluronic acid(1 α 2)D-xylopyranose(1 β 4)D-xylopyranose; this last spot also corresponded to the starting material, Acid (A). The fastest-moving acidic substance from Acid (B) corresponded to D-glucuronic acid; the intermediate substance was unknown, but was assumed to be an aldobiouronic acid; the slowest moving substance corresponded to the starting material, Acid (B).

A plot of the R_{m} values of the acidic products from the two partial acid hydrolyzates versus the number of monomeric units (suspected) in each product is shown in Fig. 4. Also plotted in this figure are the R_{m} values of the acids found in the partial acid hydrolyzate of sapote gum (see Fig. 3, p. 23). The curves which represent the hydrolysis products from Acids (A) and (B) can be considered identical with the lower portion of the two curves which represent the hydrolysis products from sapote gum. [Points e and e' corresponded to 4-O-methyl-D-glucuronic acid, points f and f' to 4-O-methyl-D-glucopyranosyluronic acid(1 α 2)D-xylopyranose, points A and A' to 4-O-methyl-D-glucopyranosyluronic acid(1 α 2)D-xylopyranose(1 β 4)D-xylopyranose, points r and r' to D-glucuronic acid, and points B and B' to Acid (B); therefore, the noncoincidence of the curves is due to experimental error.] Apparently, hydrolysis of Acids (A) and (B) produced, in either case, D-xylose, a monomeric uronic acid, and an aldobiouronic acid, providing additional evidence that Acids (A) and (B) were probably aldotriouronic acids as had been assumed previously.

Specific Rotation

A trace of ammonium hydroxide, which is known to increase the rate of mutarotation of sugars, was added to solutions of Acids (A) and (B), and the specific rotations $[\alpha]_{\text{D}}^{25}$ were determined. The specific rotation of Acid (A) was $+60^{\circ}$ (c ,

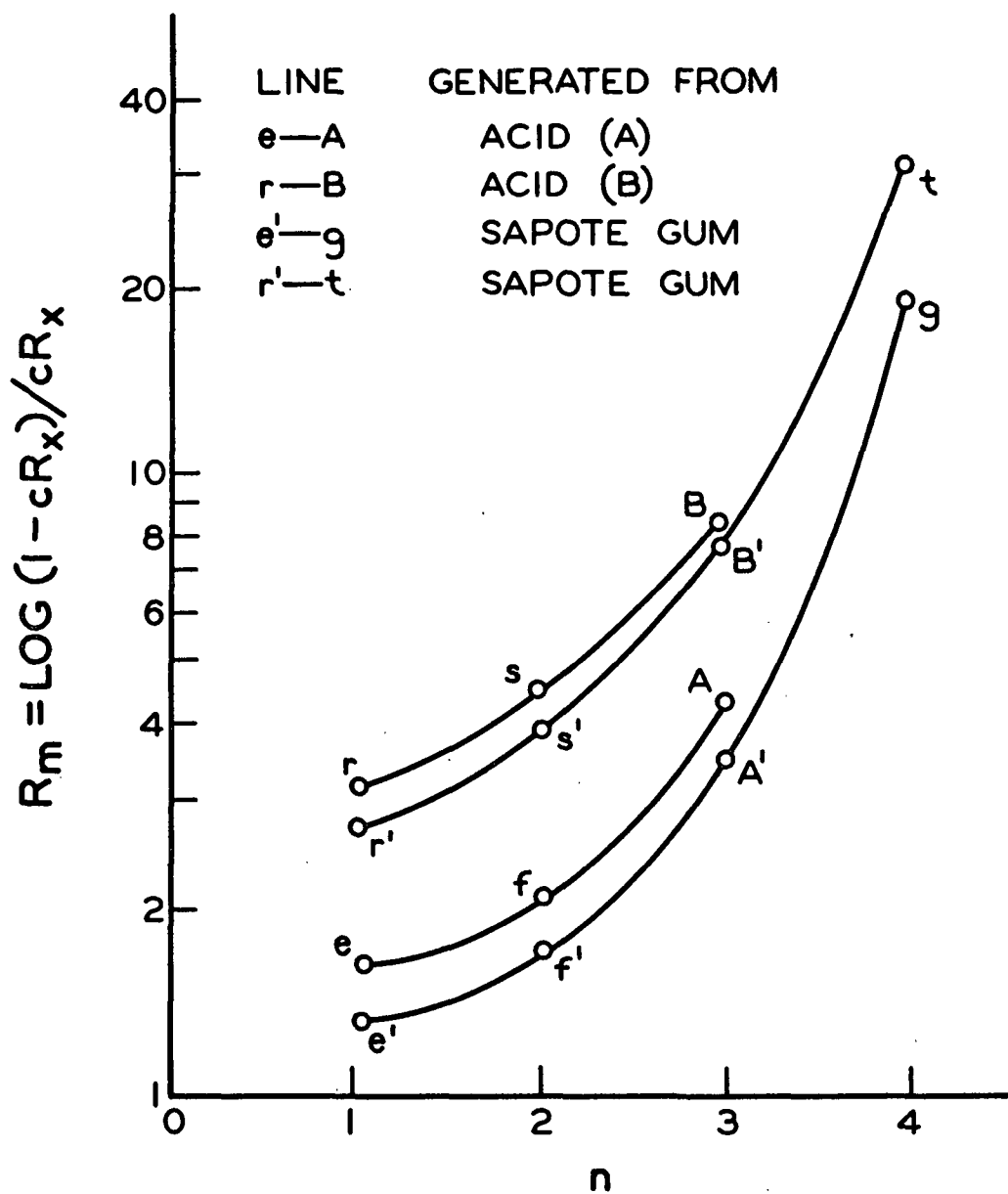


Figure 4. Chromatographic Mobility of Acids from Partial Acid Hydrolyzates of Acids (A) and (B)

0.0249 in water) and that of Acid (B) was +53° (c, 0.0259 in water). The specific rotation of Acid (A) agreed reasonably well with the reported range of +49° to +59° for 4-O-methyl-D-glucopyranosyluronic acid (1 $\xrightarrow{\alpha}$ 2)D-xylopyranose(1 $\xrightarrow{\beta}$ 4)D-xylopyranose (15). Since Acid (B) was suspected of being D-glucopyranosyluronic acid(1 $\xrightarrow{\alpha}$ 2)D-xylopyranose(1 $\xrightarrow{\beta}$ 4)D-xylopyranose, and this acid is not known to have been identified previously, no direct comparison of the value +53° could be made. However, the specific rotation of D-glucopyranosyluronic acid(1 $\xrightarrow{\alpha}$ 2)D-xylopyranose has been reported as +88° to +98° (15) and that of 4-O-methyl-D-glucopyranosyluronic acid(1 $\xrightarrow{\alpha}$ 2)D-xylopyranose has been reported as +95° to +100° (15). Since these two aldobiouronic acids have a well-defined relationship with the two aldotriouronic acids (if the suspected identities of these are correct), a reasonable prediction is that the specific rotation of Acid (B) should be similar to, although perhaps slightly lower than, that of Acid (A). Thus, +53° for Acid (B) compares reasonably well with the range of +49° to +59° for 4-O-methyl-D-glucopyranosyluronic acid(1 $\xrightarrow{\alpha}$ 2)D-xylopyranose(1 $\xrightarrow{\beta}$ 4)D-xylopyranose.

Since the specific rotation of Acid (B) is similar to the known specific rotation of 4-O-methyl-D-glucopyranosyluronic acid(1 $\xrightarrow{\alpha}$ 2)D-xylopyranose(1 $\xrightarrow{\beta}$ 4)D-xylopyranose, an evaluation by the rules of isorotation suggested by Hudson (16) indicates that, if Acid (B) is found to have the structure D-glucopyranosyluronic acid(1 \rightarrow 2)D-xylopyranose(1 \rightarrow 4)D-xylopyranose, the configurations of the two glycosidic linkages in Acid (B) must be dissimilar. Two α linkages in Acid (B) would be expected to result in a specific rotation which would be much larger than the specific rotation of 4-O-methyl-D-glucopyranosyluronic acid(1 $\xrightarrow{\alpha}$ 2)D-xylopyranose-(1 $\xrightarrow{\beta}$ 4)D-xylopyranose. Conversely, two β linkages in Acid (B) would be expected to result in a much smaller specific rotation. The small contribution made by a single methoxyl substituent to the specific rotation of one of the aldotriouronic acids can be ignored in this comparison.

Melting Point

Acid (A) melted at 182-184°C. and Acid (B) at 194-197°C. Melting points from 180 to 187°C. have been reported for 4-O-methyl-D-glucopyranosyluronic acid(1 $\xrightarrow{\alpha}$ 2)D-xylopyranose(1 $\xrightarrow{\beta}$ 4)D-xylopyranose (15). There is, of course, no value in the literature for the melting point of the previously unreported D-glucopyranosyluronic acid(1 $\xrightarrow{\alpha}$ 2)D-xylopyranose(1 $\xrightarrow{\beta}$ 4)D-xylopyranose, but 194-197°C. is a reasonable value. The acid without a methoxyl group might well have a higher melting point than the analogous acid with a methoxyl group. Without the methoxyl group, more hydroxyls would be available for hydrogen bonding between molecules. Also, the absence of a methoxyl group might result in more "efficient" orientation in the crystalline form, thereby increasing the melting point.

Equivalent Weight

Small portions of Acids (A) and (B) were subjected to paper electrophoresis with 0.1M sodium borate in the manner described by Bearce (17). A potential of 350 volts was applied for 4 hours. The acids were removed from the electrophoretograms in the manner previously described for preparative paper chromatograms.

The equivalent weights of these two acids and of Acid (K) were determined by potentiometric titration (Table IV). The equivalent weights of Acids (A) and (K) were very similar to the calculated equivalent weight of 4-O-methyl-D-glucopyranosyluronic acid(1 $\xrightarrow{\alpha}$ 2)D-xylopyranose(1 $\xrightarrow{\beta}$ 4)D-xylopyranose plus three molecules of water of crystallization. The equivalent weight of Acid (B) was very similar to the calculated equivalent weight of D-glucopyranosyluronic acid(1 $\xrightarrow{\alpha}$ 2)D-xylopyranose-(1 $\xrightarrow{\beta}$ 4)D-xylopyranose plus two molecules of water of crystallization.

The chief value of the equivalent weights determined by titration lies in their indication that the materials titrated were probably aldotriouronic acids. The accuracy of the titrations was not great enough to permit valid conclusions

regarding the number of molecules of water of hydration present. For instance, a difference of less than 2% in the experimentally determined equivalent weight of Acid (B) would have caused this equivalent weight to be nearer the equivalent weight of D-glucopyranosyluronic acid($1 \xrightarrow{\alpha} 2$)D-xylopyranose($1 \xrightarrow{\beta} 4$)D-xylopyranose plus three molecules of water of crystallization rather than two.

TABLE IV
EQUIVALENT WEIGHTS BY POTENTIOMETRIC TITRATION

Acid	Equivalent Weight by Titration	Theoretical Equivalent Weight			
		+0 H ₂ O	+1 H ₂ O	+2 H ₂ O	+3 H ₂ O
(A)	520	472	490	508	526
(K)	523				
(B)	497	458	476	494	512

X-ray Diffraction

A few milligrams of Acid (A) were dissolved in two drops of water (in a round-bottom flask) to make a thick sirup. A drop of n-butanol was added, and the flask was placed on a rotary vacuum evaporator. The sirup was kept under vacuum at room temperature for about one-half hour, and then a few crystals of Acid (K) were placed in the flask. After about two hours the sirup was very thick and brown with a large amount of small crystals mixed throughout. The sirup was placed on a porous porcelain plate in a high-humidity atmosphere. After a few minutes the plate was placed over Drierite in a vacuum desiccator. The next morning the crystals were appreciably whiter. The plate was exposed briefly to a high-humidity atmosphere and again was placed in the vacuum desiccator. The next day the crystals were very white. X-ray diffraction patterns of these crystals and of Acid (K) were made. The two patterns were found to be identical (Fig. 5).

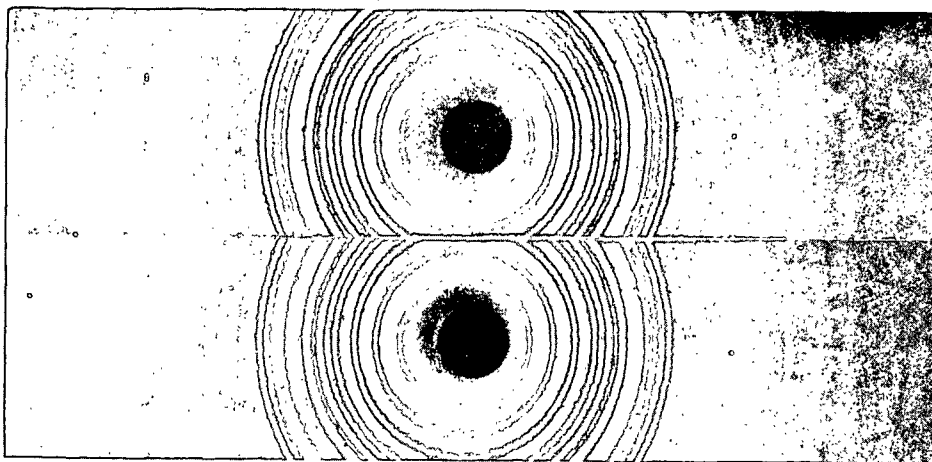


Figure 5. X-ray Diffraction Patterns of Acid (A)
(Above) (Negative 66-21) and Acid (K)
(Below) (Negative 63-1)

Infrared Absorption

The infrared absorption spectra of Acids (A) and (B) were very similar (Fig. 6 and 7). Unfortunately, it was not possible to establish the presence or absence of the single methoxyl group by this technique. Possibly background contributions from small amounts of impurities obscured the absorption band of this substituent, or the band might have been too small to have been observed readily.

STRUCTURAL INVESTIGATION OF ALDOTRIOURONIC ACIDS

A structural investigation of Acids (A), (B), and (K) was undertaken primarily to provide proof of the identity of Acid (B). In most parts of the investigation Acids (A) and (K) were processed simultaneously to provide comparisons for Acid (B) as well as to provide additional evidence for the identity of Acid (A), although the data which have already been presented probably constituted sufficient proof of the identity of Acid (A).

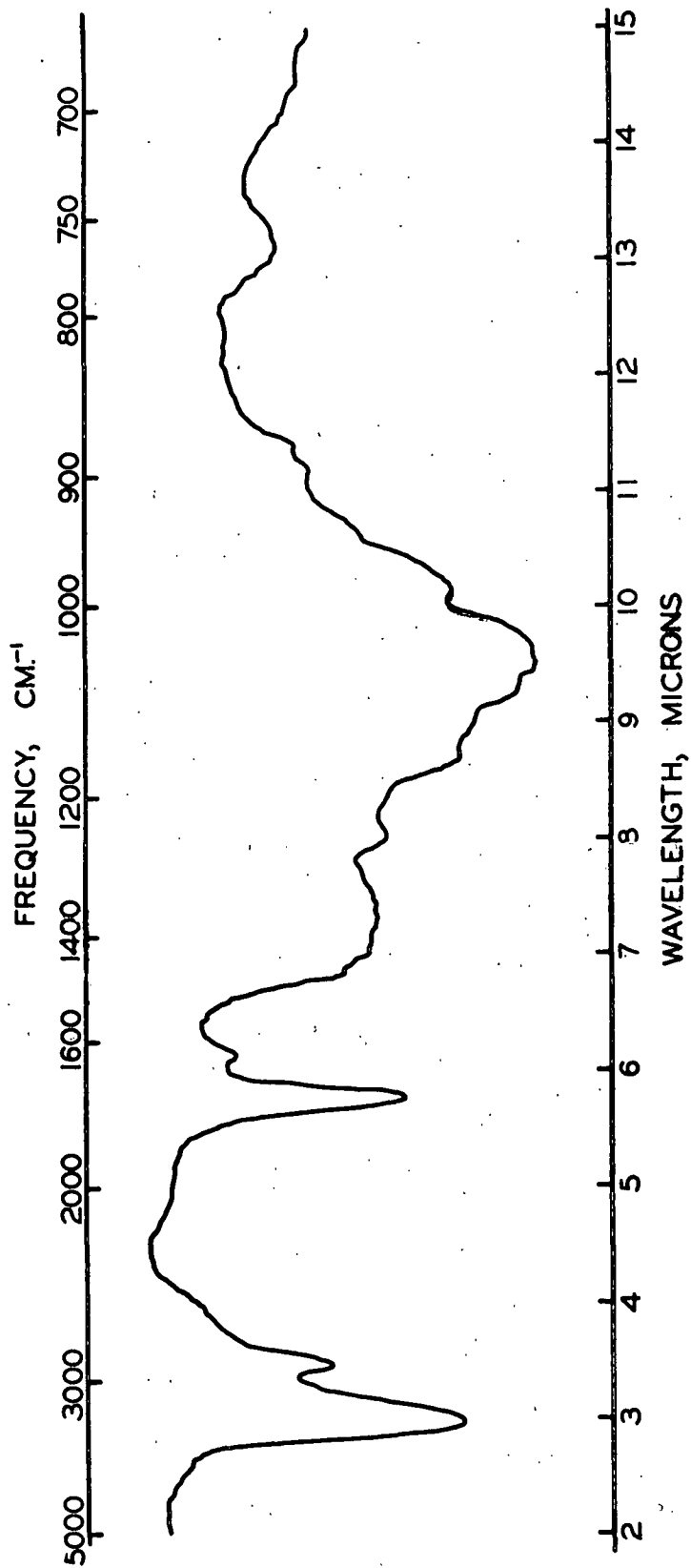


Figure 6. Infrared Absorption Spectrum of Acid (A)

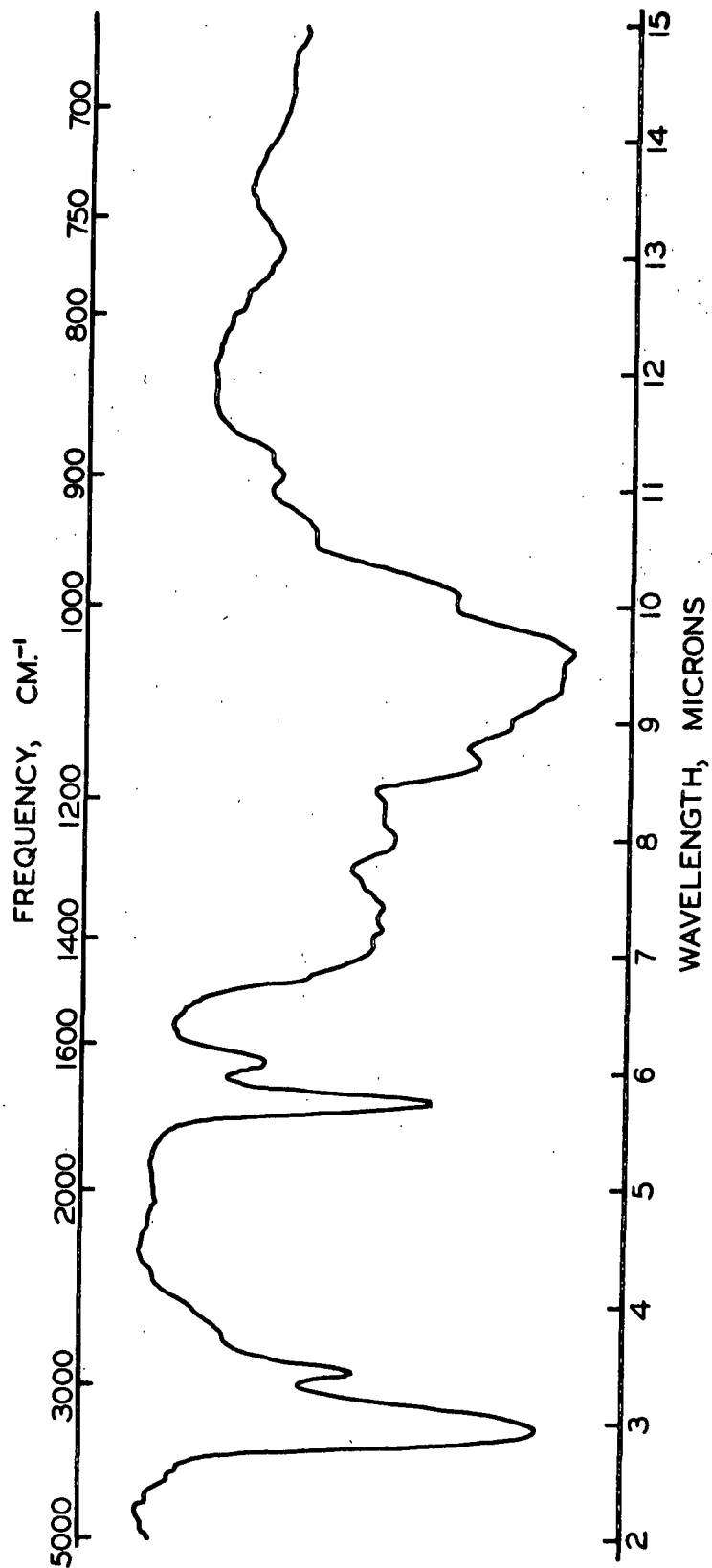


Figure 7. Infrared Absorption Spectrum of Acid (B)

IDENTIFICATION OF D-GLUCOPYRANOSYLURONIC ACID(1 \rightarrow 2)D-XYLOPYRANOSE

A 500-mg. sample of Acid (B) was dissolved in 50 ml. of 1.0N sulfuric acid, sealed in a glass ampule, and heated at 105°C. for 3 hours. The solution was neutralized with barium carbonate and was filtered. The filtrate was passed through a column of Amberlite IR-120(H⁺) ion-exchange resin and was evaporated to a thin sirup. Paper chromatography of the sirup with Solvents [A] and [B] revealed four substances, three of which corresponded to D-xylose, D-glucuronic acid, and unreacted Acid (B). The fourth substance was acidic, and its migration rate was between those of D-glucuronic acid and Acid (B). This product was assumed to be an aldobiouronic acid.

The sirup was placed on three eight-inch-wide sheets of Whatman 3-mm. chromatography paper. The chromatograms were developed with Solvent [B], dried, and inspected by the peel-strip method. Strips containing the suspected aldobiouronic acid were cut out and eluted with water. The eluate was passed through a column of Amberlite IR-120(H⁺) ion-exchange resin and was concentrated to a sirup (226 mg.) which represented 68.5% of the theoretical yield of aldobiouronic acid.

The aldobiouronic acid was identified by the formation of the crystalline acetylated methyl ester methyl glycoside. The sirupy acid was dissolved in 10 ml. of 0.5% methanolic hydrogen chloride. The solution was refluxed for eight hours. The methanolizate was neutralized with silver carbonate, filtered, and deionized on Amberlite MB-3(H⁺,OH⁻) ion-exchange resin. The solvent was evaporated to yield the sirupy methyl ester methyl glycoside. The glycoside was dissolved in 10 ml. of dry pyridine, and 10 ml. of acetic anhydride were added. The solution was sealed in a glass ampule and was heated at approximately 65°C. for three hours. The reaction mixture was cooled and poured into ice water whereupon a product solidified. The product was filtered and was washed thoroughly with cold distilled

water and then with methanol and with 50% methanol--ether. The product was dried, dissolved in redistilled chloroform, and precipitated by the addition of ice water. The precipitate was washed with ice water and then was air dried. The white crystalline product (186 mg.) melted at 255°C. alone or in admixture with an authentic sample of methyl-2-O-(methyl-2,3,4-tri-O-acetyl- α -D-glucopyranosiduronate)-3,4-di-O-acetyl- β -D-xylopyranoside. Previously recorded melting points of this compound are 250°C. (18) and 255-257°C. (19). The specific rotation, $[\alpha]_D^{26}$, was +100° (c, 0.053 in chloroform), compared with +101° (18) and +103° (19). The x-ray diffraction pattern of this product is shown in Fig. 8.

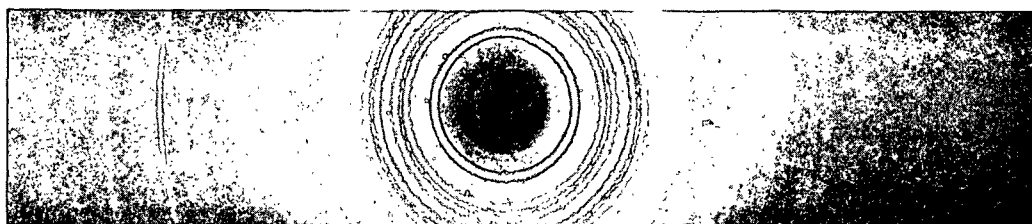


Figure 8. X-ray Diffraction Pattern of Methyl-2-O-(methyl-2,3,4-tri-O-acetyl- α -D-glucopyranosiduronate)-3,4-di-O-acetyl- β -D-xylopyranoside (Negative 66-155)

The identification of this derivative provides positive evidence that a segment of the molecule of Acid (B) was a residue of D-glucopyranosyluronic acid-(1 α 2)-D-xylopyranose. This is consistent with the suspected identity of Acid (B) as D-glucopyranosyluronic acid(1 α 2)-D-xylopyranose(1 β 4)-D-xylopyranose. The probability that the configurations of the two bonds in Acid (B) were dissimilar (based upon specific rotation) has been discussed previously. The only additional information necessary for confirmation of the identity of Acid (B) is the identity of the reducing sugar unit and the location of the glycosyl bond joining this unit to the rest of the molecule. This information was obtained by the following methylation study.

METHYLATION STUDY OF ALDOTRIOURONIC ACIDS

A methylation study of the three acids (A), (B), and (K) was undertaken primarily to determine the nature of the reducing sugar unit in Acid (B). However, it was realized that a methylation study also could provide additional evidence for the previously-identified segment, D-glucopyranosyluronic acid(1 $\xrightarrow{\alpha}$ 2)D-xylopyranose.

Sodium Borohydride Reduction

A 200-mg. portion of each of the three aldotriouronic acids was dissolved in 30 ml. of 0.1N sodium hydroxide solution which contained 4% sodium borohydride. The reaction mixtures were stirred for three hours and then were neutralized slowly (while the containers were in an ice-water bath) with 2N acetic acid. Each solution was passed through a column of Amberlite IR-120(H⁺) ion-exchange resin and subsequently was evaporated to dryness. The solid products were dissolved several times in methanol, which was evaporated each time, until all of the borate residue was removed, leaving only sirupy products.

Methylation

Each of the sirupy products was dissolved in 6.25 ml. of 4.8% sodium bicarbonate solution in flasks which were in an ice-water bath. During the next four hours 3.25 ml. of 40% sodium hydroxide solution and 3.25 ml. of dimethyl sulfate were added dropwise and concurrently. The solutions were stirred overnight. Then 1.8 g. of solid sodium hydroxide were added, and during the next 15 hr. 3.25 ml. of dimethyl sulfate were added dropwise. Again the solutions were stirred overnight, and 1.8 g. of solid sodium hydroxide and 3.25 ml. of dimethyl sulfate were added as before. Once a few milliliters of water were added to each solution to disperse sodium sulfate which was forming. The next day each solution was neutralized with 2N sulfuric acid and was extracted with 25 ml. of redistilled chloroform. The chloroform solutions were washed twice with 25-ml. portions of distilled water, then were concentrated to thick sirups.

Each of the three sirups was dissolved in 5 ml. of dry dimethylformamide. Two milliliters of methyl iodide and 0.25 g. of Drierite were added to each solution. The solutions were stirred for four hours, after which two grams of silver oxide were added during a three-hour period. The solutions were left in darkness overnight. Then 2 ml. of methyl iodide were added, and during the next three hours two grams of silver oxide were added. The solutions were stirred in darkness for three days. They were then filtered through Celite filter aid. The Celite was washed extensively with chloroform, and the chloroform washings were combined with the respective filtrates. These filtrates were each extracted with 30 ml. of chloroform, which was subsequently extracted with 25 ml. of 10% potassium cyanide solution and twice with 25-ml. portions of distilled water. The chloroform solutions were evaporated to sirups which were stored over phosphorus pentoxide in a vacuum desiccator.

The infrared absorption spectra of the acidic, methylated, nonreducing products (RA1 and RK1) from Acids (A) and (K), showed no absorption in the hydroxyl stretching region (2.85 microns). However, the spectrum of the product (RB1) from Acid (B) did have a small absorption band in this region. RB1 was again methylated with methyl iodide and silver oxide as described above. A second infrared absorption spectrum showed no absorption in the 2.85-micron region.

The absorption spectra of RA1, RB1, and RK1 are shown in Fig. 9, 10, and 11. The great deal of similarity between the three spectra is probably significant since the three products should have been identical if the suspected identity of Acid (B) was correct. The slight differences between the spectra were probably due to traces of impurities. All three of the sirups were somewhat colored; the product from Acid (B) was the darkest of the three.

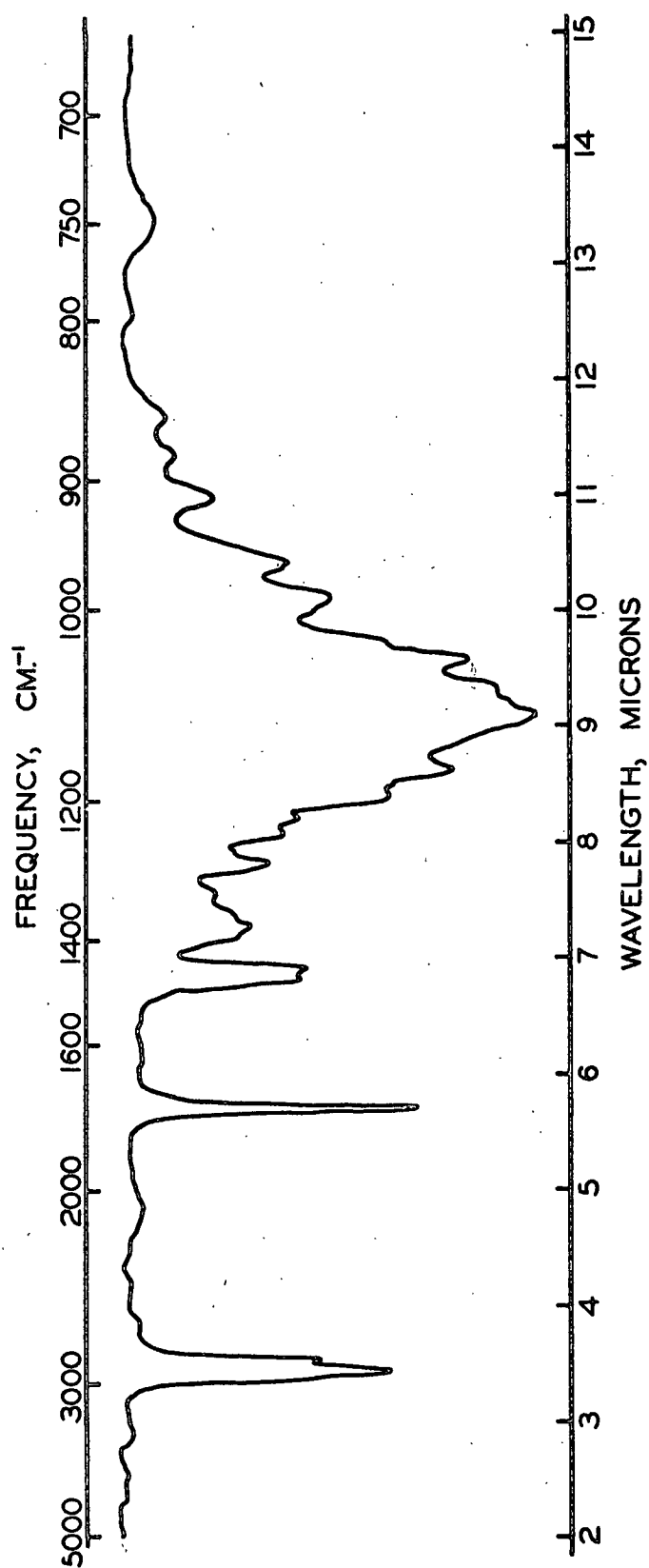


Figure 9. Infrared Absorption Spectrum of Acidic, Methylated, Nonreducing Derivative of Acid (A)

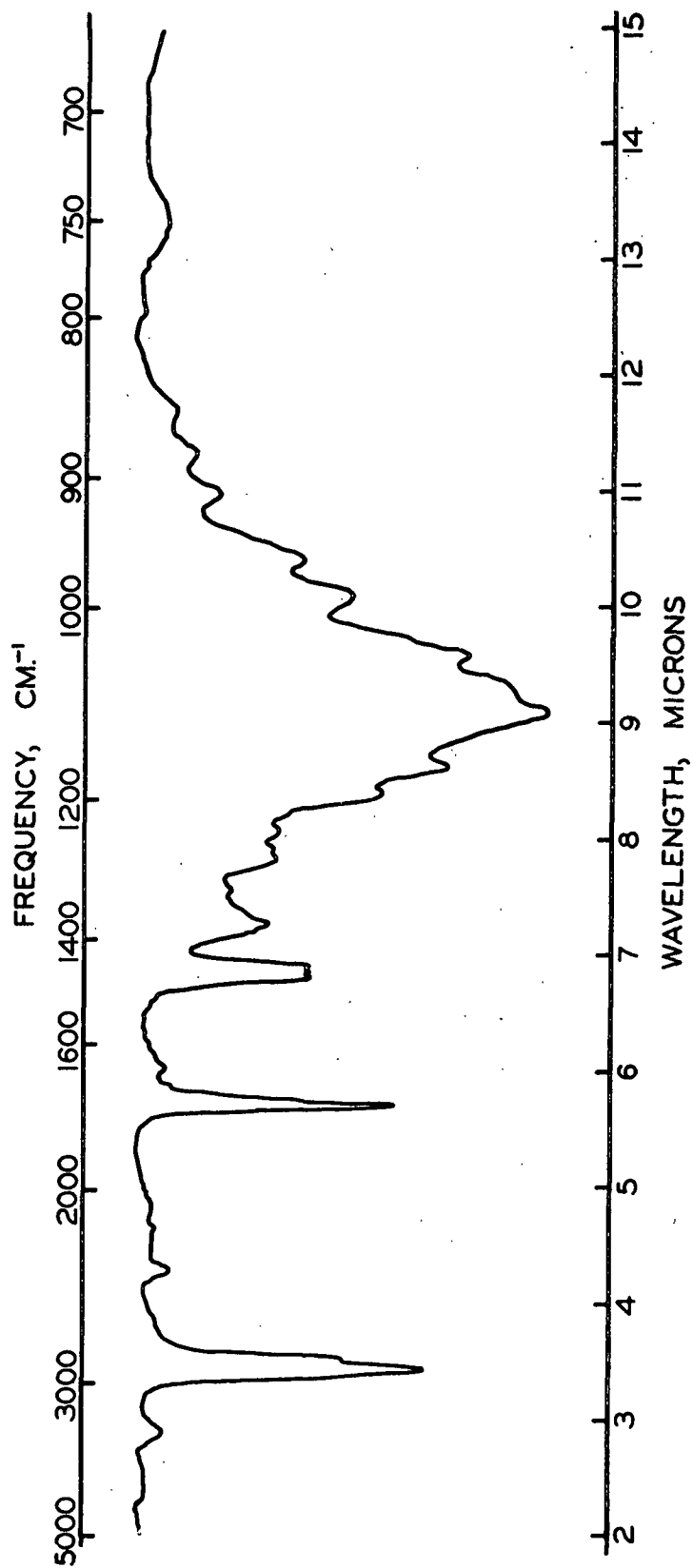


Figure 10. Infrared Absorption Spectrum of Acidic, Methylated, Nonreducing Derivative of Acid (B)

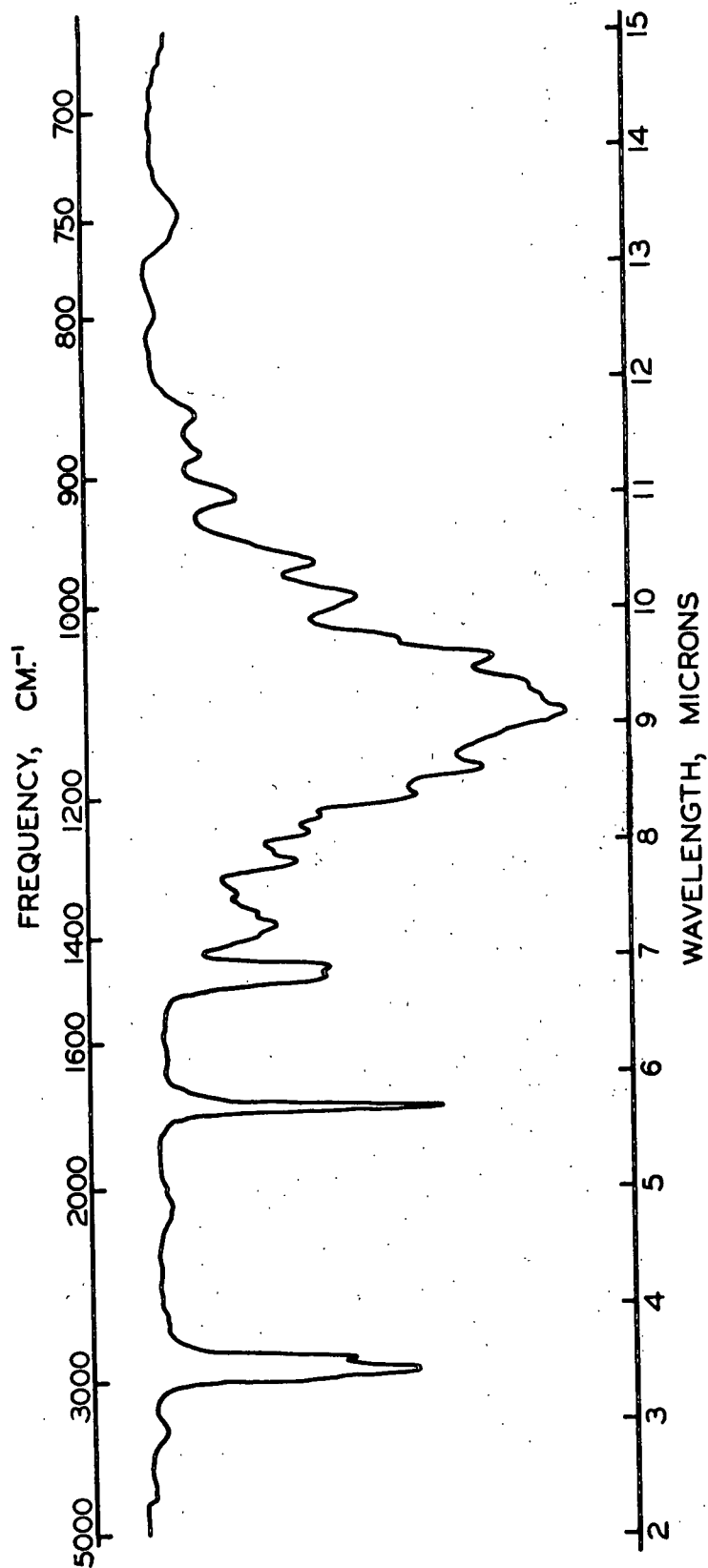


Figure 11. Infrared Absorption Spectrum of Acidic, Methylated, Nonreducing Derivative of Acid (K)

The yields of RA1, RB1, and RK1 were 139 mg., 40 mg., and 75 mg., respectively. These low yields were probably caused by emulsions which formed during the solvent extractions which followed each methylation step. These emulsions were exceedingly difficult to break, and several times it was suspected that they were not completely eliminated. It is probably significant that the smallest yield was obtained from Acid (B) and that this acid was involved in three methylation steps while Acids (A) and (K) were involved in only two.

Specific rotations ($[\alpha]_D^{25-26}$) were determined as $+38^\circ$ (c , 0.0463 in ethanol), $+38^\circ$ (c , 0.0133 in ethanol), and $+36^\circ$ (c , 0.0248 in ethanol) for RA1, RB1, and RK1, respectively. Very accurate determinations of the specific rotations were not possible for two reasons--the actual rotations measured were small (between 1.0 and 3.5°), and colored impurities absorbed some of the light passing through the polarimeter tubes, making the visual determination of the optical rotation difficult.

Lithium Aluminum Hydride Reduction

RA1, RB1, and RK1 were each dissolved in 10 ml. of dry tetrahydrofuran. To each solution 150 mg. of lithium aluminum hydride were added slowly. After the solutions were stirred for two hours, ethyl acetate was added to destroy the unconsumed hydride. Cold distilled water was added, and the solutions were filtered. The filtrates were deionized on a column of Amberlite MB-3(H^+ , OH^-) ion-exchange resin. The deionized solutions were evaporated to sirups which were stored over phosphorus pentoxide in a vacuum desiccator. These neutralized products of RA1, RB1, and RK1 were labeled RA2, RB2, and RK2, respectively.

Hydrolysis

Twenty milligrams each of RA2, RB2, and RK2 were dissolved in 5 ml. of 4% sulfuric acid. The solutions were sealed in glass ampules and were heated at $100^\circ C$. for 120 min. The solutions were then cooled and were neutralized with barium

carbonate. Barium sulfate was removed by filtration. After the filtrates were deionized on a column of Amberlite MB-3(H^+ , OH^-) ion-exchange resin, they were evaporated to sirups.

If the suspected identity of Acid (B) was correct, each of the three hydrolyzates should have contained three products:

- (1) 1,2,3,5-tetra-O-methyl-D-xylitol
- (2) 3,4-di-O-methyl-D-xylose
- (3) 2,3,4-tri-O-methyl-D-glucose

Paper Chromatography of Hydrolyzates

When the three hydrolyzates were chromatographed with water-saturated butanone there seemed to be no intersample variations in migration rate or color (with p-anisidine hydrochloride spray) of the two spots produced by each hydrolyzate. The ratio of the area of the faster spot to the area of the slower spot was approximately 0.80 for each of the hydrolyzates, which indicated that the ratios of the materials which produced the spots were probably the same in the three hydrolyzates (20, 21).

The faster spot corresponded in migration rate to a spot of authentic 2,3,4-tri-O-methyl-D-glucose. The slower spot corresponded in migration rate to a spot of authentic 2,3-di-O-methyl-D-xylose, which is known to migrate at almost the same rate as 3,4-di-O-methyl-D-xylose with water-saturated butanone (22, 23). Therefore, it can be stated with reasonable certainty that one of the spots from each hydrolyzate corresponded in migration rate to 3,4-di-O-methyl-D-xylose. [That this spot could not have been due to 2,3-di-O-methyl-D-xylose was demonstrated by gas chromatography (p. 48).]

The third expected product of hydrolysis, 1,2,3,5-tetra-O-methyl-D-xylitol, was not visible. Neither the p-anisidine hydrochloride spray nor any other available reagent is capable of detecting, on a paper chromatogram, an alditol so nearly completely methylated.

Gas Chromatography of Hydrolyzates

The three hydrolyzates were chromatographed, with nitrogen as the carrier gas, on an analytical column packed with Chromosorb P (an acid-washed diatomaceous earth) which was coated with 15% butane-1,4-diol succinate polyester. The retention times of materials leaving the column were monitored with a hydrogen flame detector.

One peak was produced from each hydrolyzate, and the retention times of the three peaks were, within experimental accuracy, identical. It was assumed that these peaks were probably due to 1,2,3,5-tetra-O-methyl-D-xylitol.

Each of the three hydrolyzates was then chromatographed on a preparative column which was similar to the analytical column except for size (1/8 in. diam. by 5 ft.). The carrier gas was helium, and a nondestructive thermal conductivity detector was used. From each hydrolyzate the material represented by the single peak was collected, by condensation, as a colorless sirup.

Identification of 1,2,3,5-tetra-O-methyl-D-xylitol

Each of the three sirups was dissolved in 10 ml. of dry pyridine. Thirty-five milligrams of recrystallized p-nitrobenzoyl chloride were added to each solution. The solutions were sealed in glass ampules and were heated at approximately 65°C. for 50 min. The solutions were kept overnight and then were neutralized with saturated sodium carbonate solution and were extracted three times with redistilled chloroform. The chloroform solutions were dried overnight over anhydrous calcium sulfate, filtered, and evaporated to dryness. The products were washed with cold dry methanol. Their melting points, alone or in admixture with one another, were 185-187°C. This melting range compares favorably with the reported range of 187-189°C. for 1,2,3,5-tetra-O-methyl-(4-p-nitrobenzoyl)-D-xylitol (24). The yields of this derivative from Acids (A), (B), and (K) were each approximately 10 mg.

The identification of this derivative, together with the identification of D-glucopyranosyluronic acid(1 $\xrightarrow{\alpha}$ 2)D-xylopyranose as a hydrolysis product of Acid (B) and evidence that the two glycosyl bonds in Acid (B) are dissimilar, is sufficient to confirm the identity of Acid (B) as D-glucopyranosyluronic acid(1 $\xrightarrow{\alpha}$ 2)D-xylopyranose(1 $\xrightarrow{\beta}$ 4)D-xylopyranose.

Methanolysis

The unhydrolyzed portions of RA2, RB2, and RK2 were each dissolved in 10 ml. of 4% methanolic hydrogen chloride. The solutions were sealed in glass ampules and kept at 105°C. for 5 hr., after which they were neutralized with silver carbonate. Silver carbonate and silver chloride were removed by filtration. The filtrates were deionized on a column of Amberlite MB-3(H⁺,OH⁻) ion-exchange resin and were evaporated to sirups. Five products were expected in each hydrolyzate:

- (1) 1,2,3,5-tetra-O-methyl-D-xylitol
- (2) methyl α -3,4-di-O-methyl-D-xylopyranoside
- (3) methyl β -3,4-di-O-methyl-D-xylopyranoside
- (4) methyl α -2,3,4-tri-O-methyl-D-glucopyranoside
- (5) methyl β -2,3,4-tri-O-methyl-D-glucopyranoside

Gas Chromatography of Methanolyzates

The three methanolyzates were inspected by analytical gas chromatography in the same manner as was described previously for the three hydrolyzates. Five peaks, the relative retention times of which are given in Table V, were obtained from each methanolyzate. All intersample variations in the retention times of these peaks were within experimental accuracy. This agreement in the retention times of these peaks is, of course, important since the three hydrolyzates were each expected to contain the same five products.

TABLE V

RELATIVE GAS CHROMATOGRAPHIC RETENTION TIMES
OF METHANOLYSIS PRODUCTS

Temperature	165°			170°			184°		
Pressure ^a	20 p.s.i.			15 p.s.i.			15.5 p.s.i.		
Starting material, acid	(A)	(B)	(K)	(A)	(B)	(K)	(A)	(B)	(K)
Peak Number									
1	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
2	1.89	1.89	1.90	1.90	1.89	1.93	1.80	1.80	1.78
3	2.22	2.24	2.30	2.36	2.28	2.41	2.14	2.14	2.13
4	3.72	3.75	3.90	4.12	4.00	4.14	3.45	3.45	3.42
5	5.40	5.48	5.62	5.58	5.75	6.07	4.85	4.84	4.75
Actual retention time of Peak 1, minutes ^b	8.1	8.1	7.9	11.3	11.9	11.0	6.4	6.5	6.4

a

Pressure of nitrogen at entrance to column; flow rate of nitrogen entering the column is proportional to pressure; flow rate within the column is a function of temperature.

b

Retention time is a function of flow rate and temperature.

The fastest-moving product in each sample had the same retention time as the 1,2,3,5-tetra-O-methyl-D-xylitol peaks from the three hydrolyzates. (This was determined by chromatographing the hydrolyzates and methanolizates consecutively under the same conditions.) The second and third peaks were probably due to methyl β -3,4-di-O-methyl-D-xylopyranoside and its α anomer, respectively. The fourth and fifth peaks were probably due to the β and α anomers, respectively, of methyl 2,3,4-tri-O-methyl-D-glucopyranoside. Bishop (25) has found that for methylated derivatives of D-glucose, D-xylose, and D-mannose, the anomer with a trans relationship between the C₁ and C₂ methoxyls has the shorter retention time.

When the paper chromatographic investigation of the three hydrolyzates was discussed (p. 44), the use of 2,3-di-O-methyl-D-xylose as a chromatographic standard for 3,4-di-O-methyl-D-xylose was described. Proof that the chromatographic spot from the three hydrolyzates could not have been due to 2,3-di-O-methyl-D-xylose was obtained by gas chromatography. A sample of fully methylated 4-O-methyl-D-glucuronoxylan was methanolized, and the methanolizate was chromatographed along with the three methanolizates described above. The strong peaks produced by methyl- α -2,3-di-O-methyl-D-xylopyranoside and its β anomer were found to have relative retention times (compared with 1,2,3,5-tetra-O-methyl-D-xylitol) of 2.16 and 2.62 under the conditions of the 165°C. column of Table V. None of the peaks from the three methanolizates described above had such retention times; therefore, the methyl glycosides of 2,3-di-O-methyl-D-xylose could not have been present in the methanolizates, and 2,3-di-O-methyl-D-xylose could not have been present in the three hydrolyzates.

DISCUSSION OF RESULTS

COMPOSITION OF SAPOTE GUM

The compositions of five nodules from each of two samples of sapote gum were determined. Some rather small intrasample variations were observed, but the compositions of the two samples were, within experimental accuracy, identical. The results of this study and the results of two other studies of the composition of sapote gum are shown in Table VI. Qualitatively, the agreement between these studies is excellent. White (3) identified partially methylated derivatives of D-xylose, L-arabinose, and D-glucuronic acid. Anderson and Ledbetter (2) identified D-xylose and L-arabinose, established the presence of a uronic acid and a methoxyl-containing uronic acid, and obtained evidence of D-glucuronic acid. In the present study D-xylose, L-arabinose, D-glucuronic acid, and 4-O-methyl-D-glucuronic acid were identified (the monomeric acids being identified as portions of oligouronic acids). Also in the present study evidence was found of the presence of a small amount of other polysaccharide material associated with sapote gum.

TABLE VI

COMPOSITION OF SAPOTE GUM

Component	Mole Ratio of Component to Uronic Anhydride		
	According to Present Study	According to Anderson and Ledbetter (2)	According to White (3)
Pentosan	--	3.4-3.6	--
Araban	1.0	0.4	0.5
Xylan	<u>2.2</u>	<u>(3.1)</u>	<u>2.0</u>
Total	3.2	3.5	2.5
Uronic anhydride	1.0	1.0	1.0
Methoxyl	0.58	0.52-0.65	--

Since different analytical techniques were employed in the three studies, it is not possible to make a rigid comparison of the quantitative results. The composition given by White is the result of a quantitative paper chromatographic analysis of the hydrolyzed reduction products of partially methanolized sapote gum methyl ether. The results attributed to White in Table VI were calculated from the ratios of partially methylated sugar derivatives given by him. The other two analyses listed in Table VI were made directly upon sapote gum. In these analyses uronic anhydride and methoxyl were determined by similar techniques. In the present study, both araban and xylan were determined by the method of Saeman, et al. (9). In the study by Anderson and Ledbetter araban was determined by the method of Neuberg (26), and xylan was calculated as the difference between pentosan and araban.

It is interesting to note that all of the major differences between the three compositions in Table VI are due to differences in the percentage of arabinose units found in the three studies. The present study and the study by Anderson and Ledbetter are in close agreement on two major points--the ratio of pentosan to uronic anhydride and the ratio of methoxyl to uronic anhydride. The only major point about which there is appreciable disagreement between these two studies is the percentage of arabinose units in the gum. Since araban was determined by different methods in the two studies, a critical discussion of this disagreement is not possible without a laboratory comparison of the two methods. However, it can be pointed out that in the present study the determination of both xylan and araban provided a check on the araban, whereas no such internal check was present in the study by Anderson and Ledbetter since they calculated xylan as the difference between pentosan and araban. Nevertheless, the possibility that the two studies involved samples of sapote gum which actually were different in araban content cannot be discounted completely.

The results obtained by White and the results obtained in the present study also differ because of a disagreement in the percentage of arabinose units in the gum. White initially determined, by quantitative paper chromatography, the following molar ratio of products from sapote gum methyl ether:

3-O-methyl-D-xylose	2.80 parts
2,3,4-tri-O-methyl-D-xylose	0.97 part
3,4-di-O-methyl-D-glucose	1.11 parts
2,3,4-tri-O-methyl-L-arabinose	
plus a possible unidentified sugar	2.12 parts

Later he determined that the previously unidentified sugar was 2,3,4-tri-O-methyl-D-glucose. He then used a Magnesol column to separate quantitatively 2,3,4-tri-O-methyl-L-arabinose and 2,3,4-tri-O-methyl-D-xylose from an unreduced mixture of hydrolysis products of sapote gum methyl ether (thus eliminating interference from 2,3,4-tri-O-methyl-D-glucose). He found 1.05 parts of the trimethyl arabinose to 0.97 part of the trimethyl xylose. He then applied this ratio to the results above to give the following molar ratio of products:

3-O-methyl-D-xylose	2.80 parts
2,3,4-tri-O-methyl-D-xylose	0.97 part
3,4-di-O-methyl-D-glucose	1.11 parts
2,3,4-tri-O-methyl-D-glucose	1.07 parts
2,3,4-tri-O-methyl-L-arabinose	1.05 parts

White then stated that the molar ratio of these products approximated, within experimental accuracy, the whole number ratio 3:1:1:1:1. From this ratio the values in Table VI were calculated. White also stated, "Again, the complexity of the evaluation and the possibility of error is emphasized." An error in the determination of the trimethyl arabinose could account for the disagreement in the composition of sapote gum as reported by White and as determined in the present study. It can be seen that, if the determination of trimethyl arabinose was too low by only a relatively small amount, the ratio of araban to uronic anhydride would be low by a much greater amount since the calculated amount of trimethyl glucose would have been too high by an amount equal to the error in the amount of trimethyl arabinose.

From the above discussion it can also be seen that, considering the complexity of White's analysis, the differences between his results and the others in Table VI are probably not as great as they might seem.

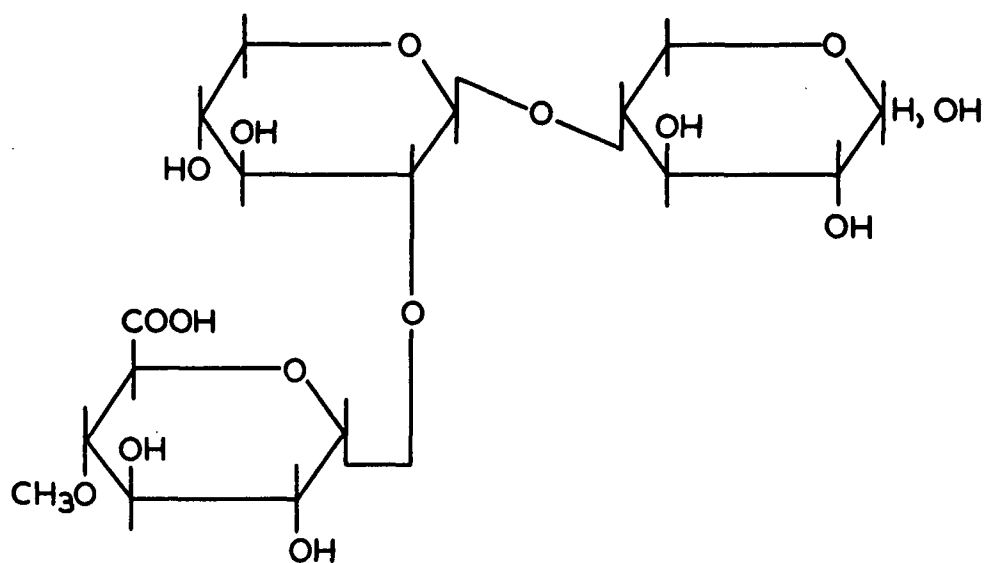
It must be emphasized that one of the samples of gum (the "old" gum) analyzed in the present study had been stored at The Institute of Paper Chemistry for a number of years and almost certainly was a portion of the sample which had been analyzed by White. If this is indeed the case, the probability that the differences between the results obtained by White and the results obtained in the present study represent real differences in the composition of sapote gum is almost nil.

In summary, it is the author's opinion, with some reservation, that the compositions of various samples of sapote gum are very similar and that apparent differences which have been reported are probably due primarily to the use of different analytical techniques.

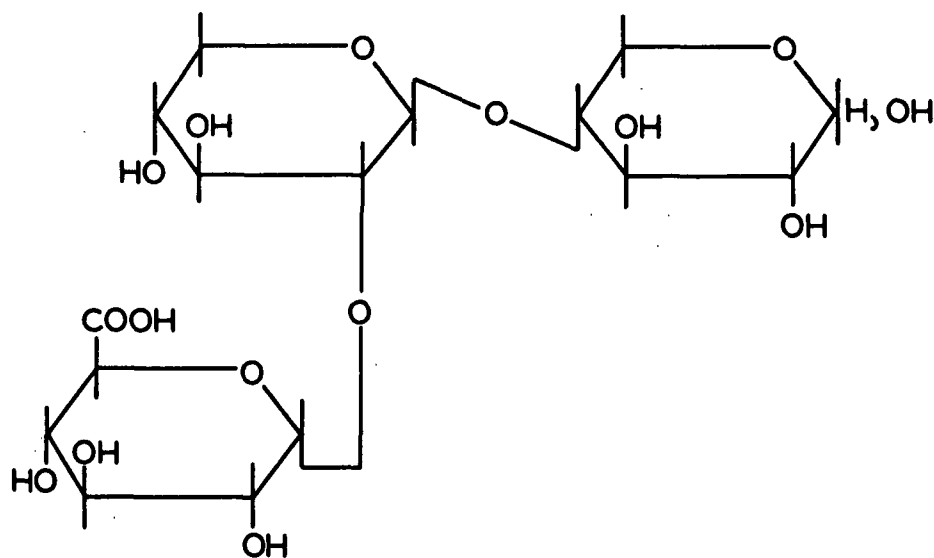
IDENTIFICATION OF TWO ALDOTRIOURONIC ACIDS

Two crystalline aldotriouronic acids (Fig. 12) were isolated from the partial acid hydrolyzate of sapote gum. Acid (A) was 4-O-methyl-D-glucopyranosyluronic acid(1 α 2)D-xylopyranose(1 β 4)D-xylopyranose. This well-known acid has been isolated previously from partial acid hydrolyzates of several polysaccharides (23,24,27-34). Acid (B) was D-glucopyranosyluronic acid (1 α 2)D-xylopyranose--(1 β 4)D-xylopyranose. Apparently, this acid has not been reported previously either from a natural source or from synthesis.

The quantity of Acid (A) which was isolated represented approximately 3.7% of the total weight of the purified gum, and the quantity of Acid (B) represented approximately 1.5% of the total weight of the gum. Together Acids (A) and (B) represented approximately 8.5% of the total uronic anhydride in the gum. Considering



ACID (A): 4-O-METHYL-D-GLUCOPYRANOSYLURONIC ACID
(1- α -2)D-XYLOPYRANOSE (1- β -4)D-XYLOPYRANOSE



ACID (B): D-GLUCOPYRANOSYLURONIC ACID (1- α -2)
D-XYLOPYRANOSE (1- β -4)D-XYLOPYRANOSE

Figure 12. Aldotriuronic Acids Isolated from Sapote Gum

Acids (A) and (B) together, the isolated acids contained approximately 0.70 methoxyl group per uronic acid unit, which, considering the inevitable mechanical losses involved, is reasonably similar to the ratio of 0.58 methoxyl group per uronic acid unit in the purified gum.

In Table VII is presented a comparison of several physical and chemical properties of Acids (A) and (B) and of an authentic sample of 4-O-methyl-D-glucopyranosyluronic acid(1 α 2)D-xylopyranose(1 β 4)D-xylopyranose, Acid (K). In Table VIII is presented a comparison of the acidic, methylated, nonreducing derivatives of Acids (A), (B), and (K) and also a comparison of the neutral, methylated, nonreducing derivatives of these three acids. All of the essential data for the identification of Acids (A) and (B) are presented. Several of the items related to each of the acids must be considered as circumstantial, but nevertheless they are important subsidiary contributions to the identifications.

ACID (A)

The equivalent weight of Acid (A) was determined, by potentiometric titration, as 520. The calculated equivalent weight of an aldotriouronic acid composed of one hexuronic acid unit, two pentose units, and one methoxyl group is 472 with no water of hydration, and 490, 508, and 526 with one, two, and three molecules of water of crystallization, respectively. The experimentally determined value of 520 is not sufficiently accurate to permit a conclusion to the effect that Acid (A) contained three molecules of water of crystallization; however, the value of 520 proves beyond reasonable doubt that Acid (A) was an aldotriouronic acid as opposed to an aldobio- or aldotetrauronic acid.

Comparisons between Acids (A) and (K) in regard to melting point, specific rotation, chromatographic mobility, infrared absorption, and apparent hydrolysis products provide evidence that the two acids were identical. Additional evidence

TABLE VII

COMPARISON OF ALDOTRIOURONIC ACIDS

Points of Comparison	Acid (A)	Acid (K)	Acid (B)
Equivalent weight	520	523	497
Melting point	182-184°C.	180 to 187°C. (15)	194-197°C.
Specific rotation	+60°	+48 to +59°C. (15)	+53°
Chromatographic mobility (R_x in Solvent [B])	0.58	0.58	0.30
Infrared absorption spectrum	Similar		Similar
X-ray diffraction pattern	(A) and (K) were identical.		--
Paper chromatographic inspection of complete acid hydrolysis products	(A) and (K) identical: Two spots corresponded in migration rate and color to 4-O-methyl-D-glucuronic acid and D-xylose.		Two spots corresponded in migration rate and color to D-glucuronic acid and D-xylose.
Paper chromatographic inspection of partial acid hydrolysis products	(A) and (K) identical: Three spots corresponded in migration rate and color to 4-O-methyl-D-glucuronic acid, D-xylose, and 4-O-methyl-D-glucopyranosyluronic acid(1 $\frac{1}{2}$ 2)D-xylose.		Two spots corresponded in migration rate and color to D-glucuronic acid and D-xylose; a third, acidic, spot was present. This substance was identified as D-glucopyranosyluronic acid-(1 $\frac{1}{2}$ 2)D-xylopyranose.

TABLE VIII

COMPARISON OF DERIVATIVES OF ALDOTRIBOURNIC ACIDS

Points of Comparison	Acid (A)	Acid (K)	Acid (B)
Infrared absorption spectrum of acidic, methylated, nonreducing derivative	No apparent differences between any of the spectra.		
Specific rotation of acidic, methylated, nonreducing derivative	+38°	+36°	+38°
Paper chromatographic inspection of hydrolysis products of neutral, methylated, nonreducing derivative	(A), (K), and (B) identical: Two spots corresponded in migration rate and color to 2,3,4-tri-O-methyl-D-glucose and 3,4-di-O-methyl-D-xylose.		
Gas chromatographic inspection of hydrolysis products of neutral, methylated, nonreducing derivative	(A), (K), and (B) identical: Each had one peak, with no intersample variation in retention time; this material was identified as 1,2,3,5-tetra-O-methyl-D-xylitol.		
Gas chromatographic inspection of methanolysis products of neutral, methylated, nonreducing derivative	(A), (K), and (B) identical: Each had five peaks, with no intersample variation in retention times; one peak corresponded to 1,2,3,5-tetra-O-methyl-D-xylitol.		

is supplied by the fact that the acidic, methylated, nonreducing derivatives of Acids (A) and (K) had apparently identical infrared absorption spectra and, within experimental accuracy, identical specific rotations. Further evidence indicating the identity of the two acids is the fact that the hydrolysis and methanolysis products of the neutral, methylated, nonreducing derivatives seemed to be identical when inspected by paper chromatography and gas chromatography. Another important point of comparison was the identical x-ray diffraction patterns produced by the two acids. These patterns, along with the supporting evidence enumerated above, are sufficient to identify Acid (A) as 4-O-methyl-D-glucopyranosyluronic acid(1 $\xrightarrow{\alpha}$ 2)-D-xylopyranose(1 $\xrightarrow{\beta}$ 4)D-xylopyranose.

ACID (B)

The identification of Acid (B) could not be made by a comparison with authentic material since apparently D-glucopyranosyluronic acid(1 $\xrightarrow{\alpha}$ 2)D-xylopyranose(1 $\xrightarrow{\beta}$ 4)D-xylopyranose has not been identified previously. The experimentally determined equivalent weight (497) indicated that Acid (B) was an aldotriouronic acid. Partial acid hydrolysis of Acid (B) yielded the known aldobiouronic acid D-glucopyranosyluronic acid(1 $\xrightarrow{\alpha}$ 2)D-xylopyranose. The identification of this aldobiouronic acid established the identities of two of the sugar units and the configuration of one of the glycosidic bonds in Acid (B). The identification of 1,2,3,5-tetra-O-methyl-D-xylitol as a product of hydrolysis of the neutral, methylated, nonreducing derivative of Acid (B) indicated that the reducing unit of Acid (B) was a D-xylose unit joined at C₄ to the rest of the molecule. The only additional information necessary for the identification of Acid (B) is the configuration of the glycosidic bond joining the two xylose units. It can be shown by the rules of isorotation suggested by Hudson (16) that this bond is in the β configuration and, therefore, that Acid (B) is D-glucopyranosyluronic acid-(1 $\xrightarrow{\alpha}$ 2)D-xylopyranose(1 $\xrightarrow{\beta}$ 4)D-xylopyranose.

The specific rotation of D-glucopyranosyluronic acid(1 $\xrightarrow{\alpha}$ 2)D-xylopyranose has been reported as +88° to +98° and that of 4-O-methyl-D-glucopyranosyluronic acid(1 $\xrightarrow{\beta}$ 2)D-xylopyranose as +95° to +100° (15). Apparently, the presence or absence of a 4-O-methoxyl group does not greatly affect the specific rotation of these two aldobiouronic acids; therefore, according to the general rules of isorotation, the presence or absence of a 4-O-methoxyl group should not greatly affect the specific rotations of the next higher homologs of these two aldobiouronic acids. Thus, D-glucopyranosyluronic acid(1 $\xrightarrow{\alpha}$ 2)D-xylopyranose(1 $\xrightarrow{\beta}$ 4)D-xylopyranose (I) and 4-O-methyl-D-glucopyranosyluronic acid(1 $\xrightarrow{\alpha}$ 2)D-xylopyranose(1 $\xrightarrow{\beta}$ 4)D-xylopyranose (II) would be expected to have similar specific rotations. Acid (B) is known to be identical to (I) except for doubt concerning the configuration of the 1 \rightarrow 4 bond in Acid (B). Acid (B) and (II) are known to have similar specific rotations; that of Acid (B) was determined as +53°; that of (II) has been reported as +49° to +59° (15). It would be expected from the isorotation rules that Acid (B) would have a much higher specific rotation than (II) if the 1 \rightarrow 4 bond in Acid (B) were in the α configuration. The fact that the specific rotation of Acid (B) is not higher than, but instead is similar to, the specific rotation of (II) indicates that the 1 \rightarrow 4 bond in Acid (B) is in the β configuration, as is the 1 \rightarrow 4 bond in (II).

Other evidence in Tables VII and VIII can be cited to support the identification of Acid (B). The specific rotation, melting point, and apparent hydrolysis products of Acid (B) differed from those of Acids (A) and (K) in manners which are consistent with the above identification of Acid (B). There seemed to be no significant differences between the infrared absorption spectra of Acids (A) and (B). Also, the apparent identity (as revealed by infrared absorption, specific rotation, paper chromatography, and gas chromatography) of the acidic methylated, nonreducing derivatives and of the neutral, methylated, nonreducing derivatives of Acids (A), (K), and (B) support the above identification of Acid (B).

RELATION OF ALDOTRIOURONIC ACIDS
TO STRUCTURE OF SAPOTE GUM MOLECULE

Several conclusions regarding the structure of the sapote gum molecule result from the identification of aldotriouronic Acids (A) and (B) as partial acid hydrolysis products.

LOCATION OF METHOXYL

Anderson and Ledbetter (2) established the presence of a methoxyl-containing uronic acid unit in sapote gum and assumed that the methoxyl group was probably located at C₄ of the uronic acid unit. They suspected, but did not prove, that the uronic acid unit was D-glucuronic anhydride. White (3) later identified methylated derivatives of D-glucose from reduced sapote gum methyl ether and thus identified the uronic acid units, but he did not investigate the location of the methoxyl groups which are present in the unmethylated gum. Identification of Acid (A) provides the first proof that sapote gum contains methoxyl groups joined to C₄ of D-glucopyranosyluronic acid units.

CONFIGURATION OF URONIC ACID-TO-XYLOSE BONDS

White (3) determined that the uronic acid units in sapote gum are joined by glycosyl 1 → 2 bonds to D-xylose units, but he did not determine the configuration of these bonds. Identification of Acids (A) and (B) indicates that all of these bonds are probably in the α configuration. Because the aldotriouronic acids which were isolated and characterized did not represent all of the uronic acid residues of the sapote gum molecule, a definite statement that all such bonds in the molecule are in the α configuration is not warranted. However, it seems probable that the bonds not actually characterized are also in the α configuration.

LOCATION OF XYLOSE-TO-XYLOSE BONDS

White (3) obtained 3-O-methyl-D-xylose from sapote gum methyl ether and assumed that these xylose units and those having uronic acid substituents at C₂ were joined by glycosyl 1 → 4 bonds in the main chain of the molecule. He assumed that nonacidic (pentose) substituents had been located at C₂ of the xylose units which yielded 3-O-methyl-D-xylose. However, without proof it could not be stated definitely that the pentose substituents were not at C₄ and that C₂ of the 3-O-methyl-D-xylose units had not been engaged in main-chain linkage. Identification of Acids (A) and (B) provides proof that attached at C₄ of at least some of the D-xylose units which do not have acidic substituents are D-xylose units which do have uronic substituents at C₂. Thus, support is given to White's assumption that the main chain consists of 1 → 4 linked D-xylose units.

CONFIGURATION OF XYLOSE-TO-XYLOSE BONDS

Identification of Acids (A) and (B) provides evidence that the glycosyl bonds which link D-xylose units in the main chain of the sapote gum molecule are probably all in the β configuration. (This statement is made with the assumption that the D-xylose units in the two aldotriouronic acids were originally part of the main chain, an assumption which has yet to be proven.) That all such bonds definitely exist in the β configuration cannot be stated because the xylose-to-xylose bonds in the aldotriouronic acids did not represent all such bonds in the gum molecule.

COMMENTS CONCERNING THE PROPOSED STRUCTURE OF THE SAPOTE GUM MOLECULE

Reference was made previously (Fig. 1, p. 9) to a proposed structural formula, in the form of a repeating unit, for the sapote gum molecule. This repeating unit was suggested by Smith and Montgomery (1) and was based primarily upon the results

obtained by White (3) and somewhat upon the results obtained by Anderson and Ledbetter (2). In general, the results of the present study, as discussed to this point, are in agreement with this proposed structure and even help to substantiate portions of it which previously were only assumed. The location of the methoxyl group on C₄ of some of the uronic acid units, the α configuration of the uronic acid-to-xylose bonds, and the glycosyl 1 \rightarrow 4 bonds which link D-xylose units in the main chain all seem to have been anticipated in the proposed structure.

As a diagrammatic device which gives a lucid representation of the presently known facts concerning the structure of the sapote gum molecule, this proposed structure is very helpful. And, indeed, this seems to be primarily the vein in which it was proposed since Smith and Montgomery (1) state that it is one of several structures which can explain the experimental facts. However, there is a strong possibility that this structure might be misleading if it is assumed to represent a definite repeating unit within the sapote gum molecule.

The initial reason for suggesting a repeating unit for sapote gum seems to have been the fact that the results of White's (3) study of sapote gum methyl ether lent themselves readily to such an interpretation. Accordingly, the proposed structure contains 2.5 pentose units per uronic acid unit, which was the ratio found by White. However, White himself emphasized the possibility of error in an analysis of such complexity as his, and the results of both the present study and the study by Anderson and Ledbetter (2) indicate that there are probably more than three pentose units per uronic acid unit in sapote gum. If this higher ratio is correct, some rather fundamental changes in the proposed structure might be required to form a tentatively acceptable repeating unit.

Another possible objection to the acceptance of the proposed structure as a repeating unit of sapote gum is that this structure cannot readily account for the

production of aldotetrauronic acids by partial acid hydrolysis. Although the presence of such acids in the hydrolyzate was not established unequivocally, paper chromatographic evidence did seem to indicate that they were present (Fig. 2, p. 21 and Fig. 3, p. 23).

The work of Bearce (17), who has isolated an aldotetrauronic acid from the partial acid hydrolyzate of a 4-O-methylglucuronoxylan, gives an indication of the structural features which probably would be necessary for the production of a significant quantity of aldotetrauronic acid from sapote gum. The sapote gum molecule would probably have to contain a xylose unit which had a uronic acid substituent but which was isolated from other uronic acid side chains to at least the extent shown in Fig. 13 (in which X represents a xylose unit and A represents a uronic acid unit).

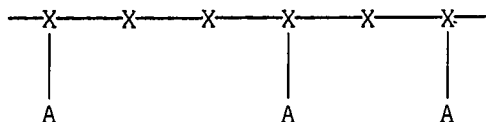


Figure 13. A Possible Arrangement Within the Sapote Gum Molecule

It is possible that such an arrangement as is shown in Fig. 13 (or some similar arrangement) could exist as a portion of a repeating unit, but its presence would make the existence of a relatively simple repeating unit seem unlikely.

It is the opinion of the author that the structural formula proposed by Smith and Montgomery, or some similar formula, is a useful device for depicting graphically present knowledge concerning the structure of the sapote gum molecule. However, it is also his opinion that changes in the proposed formula would be necessary to incorporate all of the data now available and that there is considerable doubt that such a structural formula should be regarded as a definite repeating unit.

CONCLUSIONS

Sapote gum is an acidic polysaccharide which consists of residues of uronic acid, D-xylose, and L-arabinose and of methoxyl groups in the approximate molar ratio of 1.0:2.2:1.0:0.58. All of the methoxyl is probably present as a substituent on C₄ of D-glucuronic acid residues. All of the uronic acid residues present are probably either D-glucuronic anhydride or 4-O-methyl-D-glucuronic anhydride (in the form of salts).

The compositions of carefully selected samples of sapote gum are probably very similar. Differences which have been reported in the literature are probably due primarily to the use of different analytical techniques by different investigators. Unless sufficient care is exercised, errors in determinations of the composition of sapote gum could arise from the presence of other polysaccharide material which, at least in some instances, is associated with this gum. There is also reason to believe that in some cases materials which have been sold commercially as sapote gum have been misrepresented and thus have led to erroneous findings concerning the composition of sapote gum.

Certain portions of the sapote gum molecule consist of anhydro residues of 4-O-methyl-D-glucopyranosyluronic acid(1 $\xrightarrow{\alpha}$ 2)D-xylopyranose(1 $\xrightarrow{\beta}$ 4)D-xylopyranose, and certain other portions consist of residues of D-glucopyranosyluronic acid(1 $\xrightarrow{\alpha}$ 2)-D-xylopyranose(1 $\xrightarrow{\beta}$ 4)D-xylopyranose. It is probable that such segments, or very similar segments, constitute a large percentage of the sapote gum molecule; thus, tentative generalizations concerning certain features of the structure of the molecule can be drawn from the structures of these segments. Incomplete evidence suggests that some portions of the molecule are probably so constructed that they yield aldotetrauronic acids upon mild acid hydrolysis.

There is considerable doubt that the assumption of a repeating unit for the sapote gum molecule is warranted by available data. However, it seems desirable to represent the known features of the structure of the molecule by some sort of structural diagram such as has been proposed elsewhere (1).

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APPENDIX I
PAPER CHROMATOGRAPHY

All paper chromatography was performed by the common descending solvent technique. Temperature was not controlled.

SOLVENTS

Solvent [A]: ethyl acetate (8):pyridine (2):water (1), v/v (8)

Solvent [B]: ethyl acetate (9):acetic acid (2):water (2), v/v (6)

Solvent [C]: butanone--water azeotrope (22)

DETECTION REAGENTS

Spray: p-anisidine hydrochloride (5 g.), distilled water (50 ml.), absolute ethanol (100 ml.), and n-butanol (850 ml.). Chromatograms were sprayed, dried, and heated to 105°C. for approximately five minutes (8).

Dip: Chromatograms were dipped in three solutions, consecutively, with air drying between dips and a final dip in distilled water. Solutions:
(1) silver nitrate (30 g.), distilled water (50 ml.), and acetone (950 ml.);
(2) sodium hydroxide (20 g.), distilled water (50 ml.), and absolute ethanol (950 ml.); (3) sodium thiosulfate (100 g.) and distilled water (900 ml.) (8).

PREPARATIVE CHROMATOGRAPHY

Two preparative chromatography papers were used, Whatman No. 17 and Whatman 3 mm. The material to be chromatographed was spread in a narrow band across the sheet near the upper end. Whatman 3-mm. paper was irrigated in the normal manner. A wick of Whatman No. 1 paper, previously sewed to each sheet of Whatman No. 17 paper, was used to convey solvent to the Whatman No. 17 chromatograms (Fig. 14).

PEEL-STRIP DETECTION TECHNIQUE (14)

Strips of cellophane tape were pressed longitudinally onto dried preparative chromatograms. When the strips were pulled off a thin layer of fibers adhered. This side of the strips was sprayed in the usual manner with p-anisidine hydrochloride reagent. The spotted tapes then were placed on the chromatograms to locate the invisible bands of separated materials.

ELUTION OF SUGARS

Strips containing separated materials were cut from preparative chromatograms and were irrigated with distilled water as shown in Fig. 15. The eluted product was obtained by evaporation of the collected water. A phenol--sulfuric acid test (36) of the eluate was used to determine whether elution was complete.

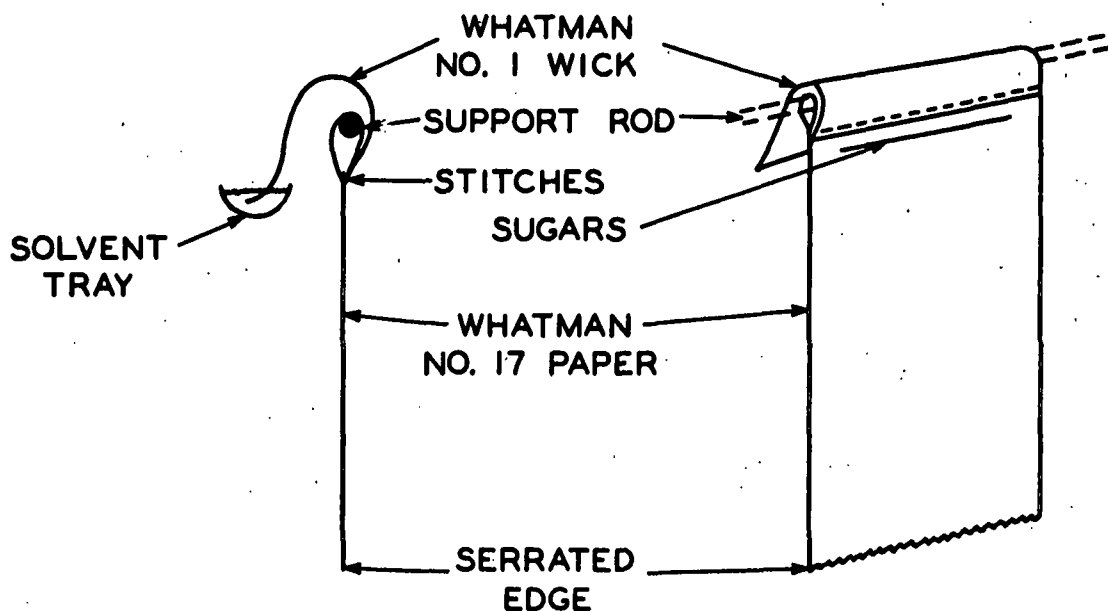


Figure 14. Preparative Chromatogram of Whatman No. 17 Paper (17)

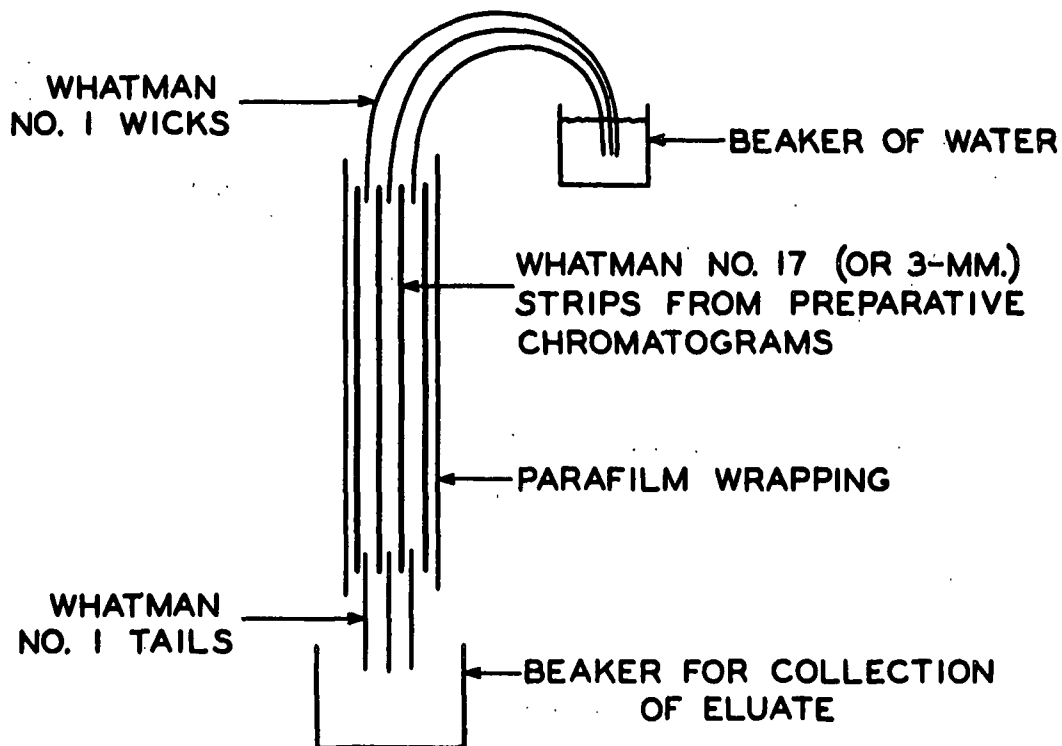


Figure 15. Preparative Chromatogram Elution Packets (Exploded Side View) (17)

APPENDIX II

LIST OF TRADE-MARKED ITEMS

Amberlite IR-45, Reagent: A weakly basic, polyamine polystyrene type ($\text{RNH}_3^+\text{OH}^-$), anion-exchange resin manufactured by Rohm & Haas, Philadelphia.

Amberlite IR-120, Reagent: A strongly acidic, sulfonated polystyrene type (RSO_3^-H^+) cation-exchange resin manufactured by Rohm & Haas.

Amberlite IRA-410, Purified: A strongly basic, quaternary ammonium polystyrene type, $\text{RN}(\text{CH}_3)_2\text{CH}_2\text{CH}_2\text{OH}^+\text{Cl}^-$, anion-exchange resin manufactured by Rohm & Haas.

Amberlite MB-3, Reagent: An indicating mixture of Amberlite IR-120 and IRA-410, fully regenerated; RSO_3^-H^+ and $\text{RN}(\text{CH}_3)_2\text{CH}_2\text{CH}_2\text{OH}^+\text{OH}^-$.

Celite Analytical Filter-Aid: An inert, acid-washed diatomaceous filter aid manufactured by Johns Manville Company, New York.

Darco G-60: An activated charcoal manufactured by Atlas Powder Company, Wilmington, Delaware.

Drierite: An anhydrous calcium sulfate desiccant manufactured by W. A. Hammond, Xenia, Ohio.

Parafilm: A flexible plastic manufactured by Marathon Division of American Can Company, Menasha, Wisconsin.